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Raul Hernando Aguirre

**Immobilization as a tool
to investigate the function
and stability of
oligomeric enzymes**

Studies on rat and beef liver arginase



Minerva Publikation München

Prof. Fernando Aguirre

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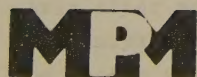
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Raul Hernando Aguirre

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der Universität Bremen als Dissertation unter dem Titel
„Immobilization as a tool to investigate the function
and stability of oligomeric enzymes: Studies on rat and
beef liver arginase“ vorgelegen. An dem Promotionsverfahren
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A b s t r a c t

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IMMOBILIZATION AS A TOOL TO INVESTIGATE THE FUNCTION AND STABILITY OF OLIGOMERIC ENZYMES: STUDIES ON RAT AND BEEF LIVER ARGINASE.

Native rat and beef liver arginases were covalently coupled to Sepharose to characterize the resulting immobilized subunits after dissociation of the matrix-bound oligomer. The dissociation of the immobilized oligomer was performed by washing with pH 2.7 buffer or with EDTA.

When immobilized rat liver arginase is extensively washed with EDTA or acid buffer, the activity recoverable in the gel is a quarter of the value of the untreated immobilized enzyme. This finding is consistent with the assumption that immobilized tetramers have been dissociated and that the remaining activity in the matrix represents the isolated, immobilized subunits. The acid-produced immobilized subunits have a K_m significantly higher than the native enzyme. Mn^{2+} associated to the acid-produced subunits is easily lost by washing with Mn^{2+} -free buffer. The immobilized tetramer does not lose Mn^{2+} by such procedure and its Mn^{2+} -affinity seems to be higher than in the free tetramer. The EDTA-produced immobilized subunits show a K_m slightly higher than the immobilized tetramer, which suggests that acid- and EDTA-produced immobilized subunits are different conformational states.

The immobilized subunits produced by both methods are capable to bind soluble subunits. That allows the application of matrix-bound subunits in order to selectively pick-up soluble subunits from a mixture of proteins, thus to purify enzyme (subunit exchange chromatography).

The results of immobilized beef liver arginase do not allow to assert with reasonable confidence that EDTA-treatment produces immobilized subunits. The activity in the matrix after acid-washing is only 5% of the original value. The enzyme responsible of this residual activity seems to be in the form of immobilized subunits as suggested by its capacity to associate soluble enzyme and to produce rat-beef-hybrids by association of rat subunits.

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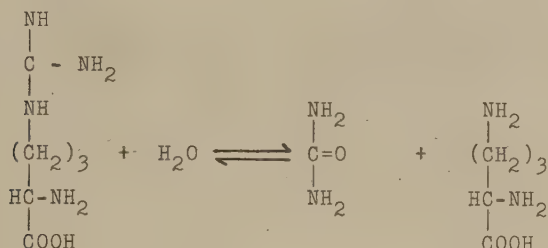
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1. INTRODUCTION

1.1 The problem and its physiological implication.

The enzyme arginase (L-arginine amidinohydrolase E.C. 3.5.3.1.) catalyzes the hydrolysis of the aminoacid arginine into the products urea and ornithine:



This reaction takes place in the metabolism of numerous species of animals and plants with different physiological significance.

Arginase was first considered to be an enzyme present only in the liver of organisms whose nitrogenous waste disposal occurs as urea (ureotelic organisms) (CLEMENTI, 1919). However, with the improvement of detection methods for enzyme activity, some arginase activity could be found in the liver of organisms where N is excreted as uric acid (uricotelic organisms).

The role of arginase in the production of urea was first understood with the elucidation of the sequence of reactions which leads to urea formation by KREBS and HANSELEIT (1932) (Fig. 1). The reactions on the left-hand side occur not only in ureotelism but in all organisms capable of synthesizing arginine. This arginine - synthesizing side may be regarded as the primitive foundation on which the urea cycle was coupled later (CAMPBELL, 1972) by the appearance of arginase activity.

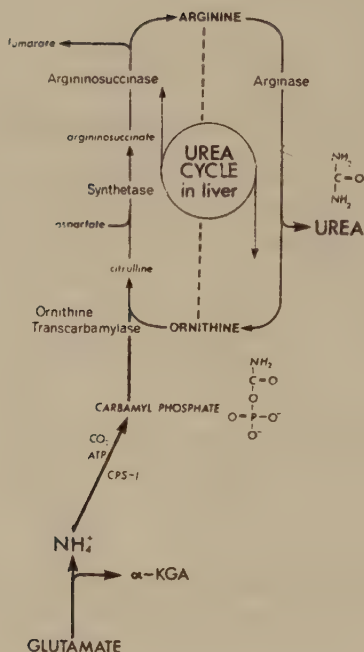


Figure 1. Pathways for the production of urea in the liver of ureotelic organisms. (Adapted from CAMPBELL, 1972).

The high level of arginase activity found in the liver of ureotelic species, in comparison with that of the other enzymes of the urea cycle (BROWN and COHEN, 1960) and the existence of this enzyme in organs that lack the capacity to synthesize arginine (kidney, brain, mammary gland, erythrocytes) would suggest a role of ureotelic arginase outside of the urea cycle. This assumption leads to the question concerning the factor determining the integration of arginase into the urea cycle. Several molecular forms of the enzyme have been characterized in ureotelic organisms but neither have structural relationships been established between these forms nor have the molecular parameters relevant to the integration of arginase(s) into the urea cycle been determined.

The ureotelic liver arginase seems to be an extramitochondrial enzyme (GABLE and LEHNINGER, 1973; MORA et al., 1965a) together with arginiosuccinic synthetase and argininosuccinase, two other enzymes involved in the urea cycle. The remaining enzymes of the cycle - ornithine transcarbamylase and carbamyl-phosphate synthetase - are located in the mitochondria (GABLE and LEHNINGER, 1973). SOBERON and PALACIOS (1976) suggest that ureotelic arginase is integrated into a functional unit which allows the enzyme to differentiate between endogenous arginine (generated from ATP, citrulline and aspartic acid in liver homogenate) and exogenous arginine. It is not clear whether this property of ureotelic arginase indicates that the enzyme is integrated in a multienzyme complex or that other forms of functional integration exists: for example, by means of control by a metabolite other than arginine, produced within the cycle, which could interact with arginase.

The molecular weight of ureotelic arginases varies between 110,000 and 140,000 for the several species analysed (HIRSCH-KOLB et al., 1970). The enzymes of rat liver (HOSOYAMA, 1972), human liver (CARVAJAL et al., 1971), beef liver (HIRSCH-KOLB et al., 1970) and rabbit liver (VIELLE-BREITBURD and ORTH, 1972) have been reported as tetramers with subunits of molecular weight ca 30,000. For rat liver-, beef liver-and human liver arginase, the subunits seem to be identical, whereas the rabbit enzyme is thought to be composed of two different kinds of subunits.

Arginase is a metal-requiring enzyme. The Mn^{2+} - dependent activity and stability was already demonstrated in 1935 by HELLMERMANN et al. Other metal ions have also been reported as activators in some extent, mainly Co^{2+} and Ni^{2+} (MAHOMED and GREENBERG, 1945; MORA et al., 1965b). The nature of the involvement of these cations in the structure and activity of the enzyme has not yet been clearly established. CARVAJAL et al.(1971) have reported that human liver arginase dissociates into inactive subunits after EDTA-effected removal of Mn^{2+} . The inactive subunits have a pI of 7.4 and reactivate and recover the tetrameric structure of pI 9.4 after addition of Mn^{2+} .

Experimental evidence (AGUIRRE, 1972) suggests that re-association and reactivation are not simultaneous processes but that an active species is formed, in the earlier stages after addition of Mn^{2+} , which has a pI close to that of the inactive subunit and a molecular weight of 27,000 , coincident with the subunits. This active form polymerizes rapidly to the tetramer. HOSOYAMA (1972) has found that rat liver arginase dissociates and is inactivated at pH 2.7 and reassociates and is reactivated by restoration of neutral pH. The reactivation kinetics show a first order process with respect to the enzyme concentration, suggesting that polymerization is not a rate-determining step in the reactivation process.

These results suggest that not only the arginase tetramer is active but also the monomer. The question concerning the activity of the subunits seems to have physiological relevance by virtue of several reports; BASCUR et al ., (1966) have found that CM- cellulose chromatography of a semipurified preparation of human liver arginase renders two peaks. About 90% of the activity is concentrated in the peak identified as a tetramer (CAR-VAJAL et al., 1971). The other peak is similar to the form obtained in the earlier stages of Mn^{2+} reactivation (AGUIRRE,1972) and thus the active monomer, at least under the criteria of ion exchange chromatography, molecular filtration and ornithine inhibition pattern. It is interesting to note that the purified tetramer does not show spontaneous dissociation, whereas ion exchange re-chromatography of the form considered to be a monomer reveals the tendency of this species to associate (AGUIRRE,1972). This situation could indicate that the equilibrium association-dissociation is somehow controlled by a ligand whose dilution causes subunit polymerization. No further information is available about the nature of this factor affecting the association equilibrium. There is, however, enough information indicating that ureotelic arginases from different sources exhibit different stabilities in the presence of certain dissociating factors.

HIRSCH-KOLB et al. (1970) have observed that beef liver arginase dissociates into subunits when the enzyme is chromatographed on Sephadex G-200. Conversely, DAHLING and POREMBSKA (1977) claim that this enzyme does not dissociate even after treatment with EDTA. In each case the enzyme was purified using rather different methods and it is possible that these differences might account for the presence or absence of an association-controlling factor. Sephadex chromatography of dog liver arginase shows a wide peak of molecular weight at about 60,000, suggesting an association-dissociation equilibrium in the column rather than a unique species of this molecular weight. Monkey and pig liver arginase reveals at least two molecular forms of different pI (HIRSCH-KOLB et al., 1970). SOROF and KISH (1969) have detected small but significant amounts of arginase of about 30,000 molecular weight (subunits) by gel filtration of crude preparations of rat liver arginase.

The assumption that active subunits and tetramers might exist simultaneously raises the question concerning the role of both forms. In this respect some observations provide evidence for the participation of arginase in functions other than the urea cycle. CURRIE (1978) has found that when rodent macrophages are exposed to stimuli, such as bacterial lipopolysaccharide or zymosan, they develop cytotoxicity for some kinds of tumor cells by releasing arginase. This cytotoxic role of arginase remains unclear, but it seems reasonable to suspect that the depletion of arginine in the medium specifically affects tumor cells owing to their elevated requirement for this amino acid. Although the addition of beef liver arginase mimics the cytotoxic effect of the released macrophage arginase, it is not known whether this tumor-cell-killing enzyme and the arginase involved in the urea cycle are the same molecular form.

The facts presented above substantiate the importance of studying the properties of the active monomer and to compare them with those of the tetrameric form as well as to further investigate the equilibrium of association-dissociation of the oligomer and the factor governing this equilibrium.

1.2 Enzyme immobilization: a necessary tool

A critical point in the characterization of the active monomer of ureotelic liver arginase is to obtain it as a stable molecular species. Oligomeric enzymes can be dissociated by treatment with 8M urea, guanidine chloride, detergents (KLOTZ et al., 1975) and in the case of arginase, by EDTA and low pH. All these treatments render inactive subunits. When the conditions of reactivation are set up the enzyme also reassociates, so that if an active monomer is previously formed, it soon polymerizes. This represents a serious limitation in the characterization of the active subunits since their properties are changing by polymerization.

An attractive approach to avoid polymerization was introduced by CHAN (1970). It consists in the attachment of the oligomeric enzyme to an insoluble matrix via a single subunit and subsequently the removal of the other, not covalently bound, subunits. If the covalently attached subunits are widely separated from another and the matrix is relatively rigid, it is possible to investigate the properties of isolated subunits under reactivation conditions which prohibit subunit interactions. Re-assembly of immobilized oligomers should also be possible by mixing soluble and immobilized subunits and subsequent restoring the associating conditions. Figure 2 summarizes the general procedure I have applied to determine the activity of arginase subunits and to characterize them.

This method has already been applied to enzymes like rabbit muscle aldolase and yeast transaldolase (CHAN, 1976). The insoluble support normally used is Sepharose, a non ionic and hydrophilic polymer structured as a highly porous matrix allowing free diffusion of macromolecules into the gel during the preparation of immobilized derivatives. Microscopic observations of immobilized fluorescent proteins indicate that under the customary conditions of immobilization, the proteins are homogeneously distributed throughout the Sepharose matrix.

CHAN (1976) has proposed a catalogue of criteria to determine the presence of immobilized subunits. The major part of these criteria is based on enzyme activity and this has the advantage

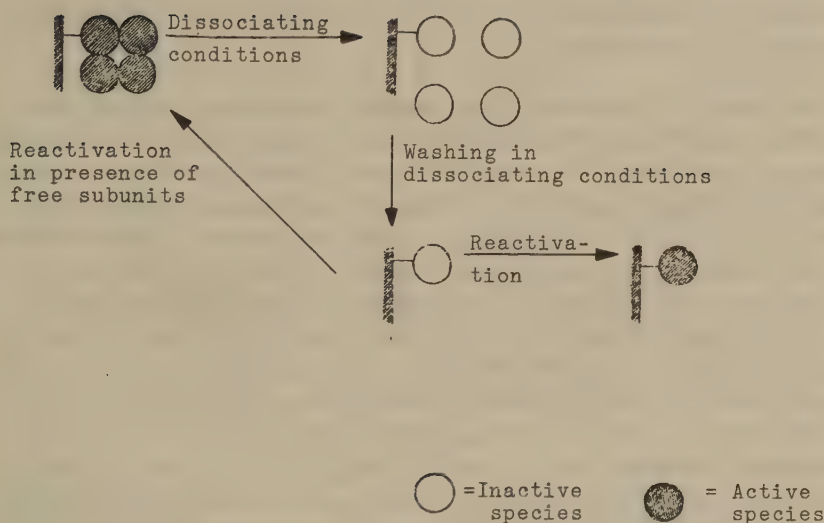


Figure 2. The reversible dissociation of immobilized oligomers. For arginase the dissociating conditions should be a low pH (2.7) or EDTA treatment. Reactivation should be carried out by neutralization or Mn^{2+} addition, respectively.

that the enzyme preparation under study does not require absolute purity. However, it is important to note that considerations about the degree of polymerization of the immobilized oligomer based on enzyme activity are reliable if the catalytic constant of the active subunit in the assay conditions is the same not only in the oligomeric state but also in the monomeric state. Obviously, that is not necessarily true for all the oligomeric enzymes with identical subunits. In the case of aldolase it seems true and the immobilized monomer represents 25% of the activity of the immobilized tetramer.

Several lines of evidence allow the assumption that these characteristics of aldolase might also be valid for human liver arginase. In fact, after the addition of Mn^{2+} to EDTA treated ar-

ginase, the reassociation of the subunits is a slower process than reactivation and no changes in the specific activity of a recently reactivated preparation, reflecting the reassociation process, are discernible.

The activity of immobilized enzymes has to be analysed cautiously in order to extrapolate to the situation of the free enzyme in solution. The main problems in this respect are related to: a) the modification of the intrinsic properties of the enzyme due to its being coupled to the matrix and b) the problems of the microenvironment of the immobilized enzyme. Part of the latter types of problem is related to the nature of the matrix and the substrate and part with the properties of the enzyme itself. In this respect, it has been observed that above a certain amount of bound enzyme, the activity no longer represents the actual concentration of the enzyme because there is not enough substrate to reach all the enzyme molecules and allow saturation thus, the immobilized enzyme becomes less effective. Studies of the effectiveness of the immobilized arginase system have to be carried out in order to overcome some of these difficulties.

1.3 Arginase-Sepharose as a model of immobilized oligomeric enzymes.

More than 200 enzymes have already been immobilized and the reports newly immobilized enzymes increases very rapidly. The majority of these enzymes are probably oligomers.

Although immobilization as a method of determining the activity of enzyme subunits were already applied 10 years ago (CHAN, 1970), many aspects of the association equilibrium of immobilized oligomers have only been partially studied. In regard to the wide field of application of immobilized enzymes, it is important to look for suitable model systems supplying as much information as possible about thermodynamic and kinetic factors controlling the association and dissociation of the matrix-bound oligomers.

Arginase fulfills some requirements which a model ought to fulfil: a) the enzyme is very stable to thermal denaturation;

in fact, a purification step consists of the incubation of the enzyme preparation for 20 min at 60° with very little loss of activity (SCHIMKE, 1964). This makes it possible to study thermal effect on the association equilibrium over a wide range of temperature using enzyme activity as an indicator. b) arginase can be dissociated under relative mild conditions- low pH or EDTA-treatment (as demonstrated for rat- and human liver arginase)- favouring a quantitative recovery of enzyme activity after establishment of the activating conditions.

A recent trend in the application of immobilized oligomers which is also interesting to explore in regard to arginase, as well, is the employment of matrix bound subunits to selectively pick up subunits from a mixture of several proteins, thus to isolate subunits. This technique is known as subunit exchange chromatography (ANTONINI et al., 1975) and has been applied to hemoglobin (ANTONINI et al., 1975), chlorophyll a/b (TAKAHASHI and GROSS, 1978), arginase (AGUIRRE and KASCHE, 1979) and recently to aryl sulfatase A (VAN ETEN and WAHEED, 1980).

2. MATERIALS AND METHODS

2.1 Reagents.

L-arginine hydrochloride, L-canavanine sulfate and Tris (Tris(hydroxymethyl) aminomethane) were purchased from Sigma Chemical Corporation (St. Louis, Mo, U.S.A.), EDTA (Ethylene dinitrotetraacetate, sodium salt, TITRIPLEX III), L-glycine and $\text{MnCl}_2 \cdot 6\text{H}_2\text{O}$ were obtained from Merck (Darmstadt, FGR). Urease (78 units/mg) was obtained from P-L Biochemicals Inc. (Milwaukee, Wisconsin, U.S.A.). Glutaraldehyde was purchased from Serva Feinbiochemica (Heidelberg, FGR). All other chemicals were of analytical grade.

2.2 Enzyme sources.

Rat liver arginase was purified according to SCHIMKE (1964) up to the ethanol precipitation step without modifications. The animals used were adult albino male rats weighing about 200g. Beef liver arginase was obtained from Sigma Chemicals Co. (Lot 47c-8055, 70 units/mg. protein) and used without further purification.

2.3 Protein determinations

2.3.1. The concentration of soluble proteins was measured by absorbance at 280 nm (WARBURG and CHRISTIAN, 1941). The arginase preparations used in this work had an optical density ratio ($A_{280}:A_{260}$) of 1.50 to 1.65 and no corrections were made for nucleic acids.

2.3.2. Direct measurements of protein content in the gel matrix were performed according to the method of GOLOVINA et al., (1977) as follows: A sample of gel suspension, containing a definite amount of gel, was mixed with four volumes of a polyethylene glycol solution containing 1g polyethylene glycol (M.W. 6000) per ml water. The suspension was shaken to homogeneity and its optical density was measured at 280 nm using an identical suspension as reference but containing Sepharose

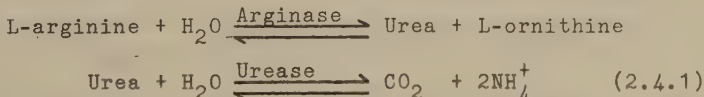
without coupled protein. Polyethylene glycol prevents settlement of the Sepharose suspension during the measurement and decreases light scattering.

2.4 Assay of the arginase activity

2.4.1. Potentiometric determination.

Several methods measuring arginase activity have been reported (VAN SLYKE and ARCHIBALD, 1946; WARD and SRERE, 1967), most of them determining product appearance or substrate depletion after the stop of the enzyme reaction. In the case of immobilized enzymes, it is desirable, however, to use a method for continuous recording of the progress of the enzyme reaction since that allows an easier estimation of initial rate conditions.

An attractive possibility was the potentiometric determination of NH_4^+ generated in a system where the measured enzyme reaction produces NH_4^+ by means of a coupled reaction catalyzed by urease:



Several analyses of coupled enzyme reactions have been carried out (McCLURE, 1969; EASTERBY, 1973; KUCHEL et al., 1975) in order to determine the assay conditions in which the formation rate of end product(s) provides a realistic evaluation of the kinetic parameters of the first enzyme-catalyzed reaction.

One of the main problems in coupled enzyme reactions consists in minimizing a transient period, previous to the steady state establishment, in which the intermediates in the sequence are accumulating and the rate of end product formation does not actually represent the activity of the enzyme under study. Usually it is assumed that the transient time is inversely proportional to V_{max} of the coupling enzyme and independent of the kinetic parameters of the first enzyme. EASTERBY (1973) has shown that for the coupling enzyme operating in the first order region, the transient time (τ_1) is given by $\tau_1 = K_1/V_1$ where V_1 and K_1 are the maximum velocity and Michaelis constant of the coupling enzyme, respectively. From this equation it is apparent

that the necessary amount of coupling enzyme to obtain a definite transition time depends on K_m and k_{cat} of the coupling enzyme. In some coupled enzyme systems it could be troublesome to reach the desired high concentration of coupling enzyme owing to its interactions with the coupled enzyme or polymerizations introducing an artifact in the determined kinetic parameters.

Urease has been investigated in this respect (KUCHEL et al., 1975) showing that the co-existence of various polymeric forms of the enzyme does not complicate the coupled reaction of arginase and that urease does not associate heterogeneously with arginase. The equation describing the progress of the arginase-urease catalyzed reaction (dNH_4^+/dt) as a function of the potential change of a NH_4^+ -sensitive electrode (dE_{obs}/dt) of Nernstian response (equation 2.4.2) can be derived as follows:

$$\text{Nernst equation at } 25^\circ : E_{obs} = E^0 + 0.059 \log(NH_4^+) \text{ (mV)} \quad (2.4.2)$$

The differentiation of this equation with respect to the time yields :

$$\frac{dE_{obs}}{dt} = 0.059 \times \frac{1}{(NH_4^+)} \times \frac{d(NH_4^+)}{dt} \quad (2.4.3)$$

According to equation 2.4.3 follows that if the experimental conditions are such that the reciprocal of ammonium ion concentration is changing much slower with time than the time differential of (NH_4^+) , the determination of $\Delta E_{obs}/\Delta t$ gives directly a measurement of the arginase catalyzed reaction rate. A test for the validity of this assumption should be simply to observe the slope of a plot of $\Delta E_{obs}/\Delta t$ as a function of the arginase concentration.

The equation 2.4.3 could be consequently related to the general rate equation describing the behaviour expected for immobilized arginase:

$$\frac{dE_{obs}}{dt} = \alpha \times \eta \times \frac{V_{max} \cdot (S)}{(S) + K_m} \quad (2.4.4)$$

α is a factor including $1/NH_4^+$ (considered as constant), η re-

presents effectiveness of the immobilized enzyme (see section 2.16, equation 2.16.1), $[S]$ is the concentration of arginine, V_{max} and K_m are the maximum velocity and Michaelis constant of immobilized arginase.

2.4.1.1 Assay conditions.

All the potentiometric determinations were carried out using a NH_4^+ selective membrane-electrode (Philips, IS 561- NH_4^+). The potential was registered using a saturated calomel electrode (Philips, Typ R 44/2-SD/1) as reference with a built-in salt bridge. The salt bridge was filled with 0.1 mole/l Tris-HCl, pH 7.5. This solution was renewed every day. The detector was a Radiometer pH meter (PHM 63, digital) in conjunction with a strip chart recorder (Servogor 5 Metrawatt, GmbH).

For a typical experiment, 20 ml of 0.1 mole/l Tris-HCl buffer pH 7.5 were filled into a thermostated reaction vessel and the solution was stirred with a motor-driven mixer. The electrode was immersed in the assay buffer and the following solutions were added successively: 2 ml L-arginine (invariable concentrations according to the experiment) in 0.1 mole/l Tris HCl pH 7.5, 0.2 ml of 1 mole/l $MnCl_2$, 0.05 ml of 0.05 mole/l NH_4Cl . After the attainment of a constant potential, 0.5 ml of 2 mg/ml urease in 0.1 mole/l Tris-HCl pH 7.5, were added and the background potential was recorded until a constant value was obtained. Normally this occurs five minutes after the addition of urease. Finally arginase was added in variable amounts according to the experiment and the potential variation was recorded. All measurements were carried out under constant stirring at a speed high enough to prevent dependence of the reaction rate on stirring speed.

The additions of NH_4Cl in a concentration of 1.12×10^{-4} mole/l diminishes in some degree the sensitivity of the method but considerably improves its accuracy providing a rapidly attainable constant background potential. As previous studies indicate (KUCHEL et al., 1975), NH_4Cl does not inhibit urease in such a concentration.

Prior to a new measurement, the electrodes were washed several

times with bidestilled water and subsequently with 0.1 mole/l Tris-HCl, pH 7.5, to a constant potential. Between measurements the electrodes were stored in 0.1 mole/l Tris-HCl, pH 7.5, at room temperature. The ion-sensitive membrane of the NH_4^+ -electrode was changed every 3 months.

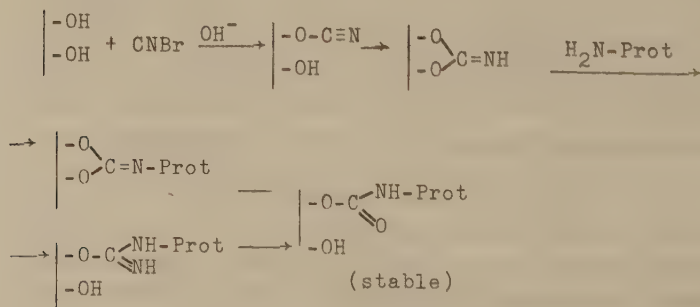
2.4.2. Colorimetric determination.

Occasionally arginase activity was determined as described by CABELLO et al. (1961): 0.6 ml of an appropriate amount of arginase in 0.1 mole/l Tris-HCl, pH 7.5, 0.01 mole/l MnCl_2 , were incubated for 5 minutes at 37° and 0.4 ml of 0.5 mole/l L-arginine, pH 8.5 were added. 10 min after substrate addition, 0.2 ml of 24% metaphosphoric acid were added to stop the reaction. The liberated urea was determined with α -isonitrosopropiophenone.

2.5. The immobilization of arginases.

The procedure of enzyme immobilization to an insoluble agarose matrix (Sephargose) is the method described by AXEN et al., (1967) with slight modifications.

This method is based on the reaction between cyanogen bromide with the hydroxyls of the polysaccharide matrix to give reactive imidocarbonates which can react with amino groups as ϵ -amino of lysine residues of proteins. The sequence of reaction can be summarized as follows:



The following description of the immobilization procedure

accounts either for the coupling of rat liver arginase or for beef liver arginase to Sepharose 4B and CL 2B.

2.5.1 Matrix activation.

A given volume of swollen Sepharose (usually 100 to 200 ml) was repeatedly washed with water in a glass filter. The gel was then suspended in an equivalent volume of BrCN solution containing 20mg BrCN per ml gel and left to react at room temperature for 10 min under gentle stirring. During the course of the reaction, pH 11 was maintained constantly by addition of 5N NaOH. The reaction mixture was then transferred to a glass filter and washed several times with water and subsequently with phosphate buffer, $I=0.5$, pH 7.0 , to constant pH.

2.5.2 Arginase coupling.

The activated Sepharose was suspended in an equivalent volume of potassium phosphate buffer $I=0.5$, pH 7.0 , and arginase dissolved in approximately 10 ml phosphate buffer was added. The suspension was shaken about 15 hours at room temperature and transferred to a glass filter and washed several times with phosphate buffer. The gel was suspended in an equivalent volume of the same buffer containing 0.1 mole/l ethanol amine and shaken about 15 hours at room temperature and subsequently washed several times with 1 mole/l NaCl-HCl, pH 4.5 , and thereafter with Tris-HCl $I=0.05$, pH 9.5, containing 1 mole/l NaCl, to equilibrium. Finally the gel was washed with 0.1 mole/l Tris-HCl, pH 7.5, to constant pH. All the filtrates were collected and their protein contents were determined by absorbance at 280 nm. The yield of immobilized protein was estimated by determining the difference between the added amount of protein and the amount that was washed out of the gel after the coupling procedure.

The concentration of immobilized enzyme is expressed either as enzyme units or as mg of protein per ml of settled gel. To determine the gel volume, a gel suspension approximately 1:1 gel-buffer was left to settle for 1 h in a graduated tube and the gel volume was measured.

The immobilized enzymes were stored in 0.1 mole/l Tris-HCl buffer, pH 7.5, at 4° and no activity loss was detected in a period of about 4 months. Arginases were also immobilized to CNBr-activated Sepharose 4 B ready to use as supplied by Pharmacia. This gel was swollen in 1 mmole/l HCl and washed in this solution and afterwards the gel was extensively washed with phosphate buffer, I=0.5, pH 7.0. The coupling and subsequent washing procedure were similar to the above described ones.

Reference gels were prepared by submitting Sepharose samples to the same conditions of activation and coupling but in the absence of enzyme.

2.6.1 Mn^{2+} removal from immobilized arginases.

Both, immobilized rat liver- and beef liver arginase were washed with EDTA-containing buffer in a closed circulating system. Samples of the Sepharose bound enzyme of 2-10ml gel were poured into a small jacketed column of 1 cm diameter and the in-and outlet of the column were connected with a thermostated reservoir containing 250 ml of 0.02 mole/l Tris-HCl, 0.1 mole/l EDTA, pH 8.0. (for beef arginase the EDTA concentration was 0.03 mole/l). The buffer is pumped through the column by means of a peristaltic pump as illustrated in Fig. 3.

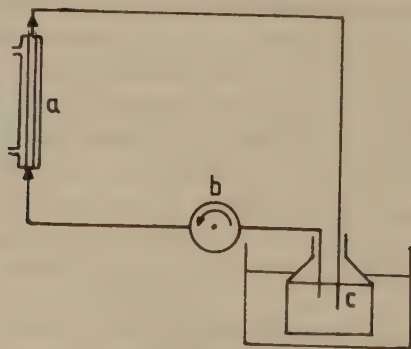


Figure 3. EDTA-washing system.

- a• Thermostated column containing the gel suspension
- b• Peristaltic pump
- c• Reservoir containing EDTA in water bath.

At given times aliquots of the gel suspension were withdrawn and washed extensively with 0.1 mole/l Tris-HCl buffer, pH 7.5 at room temperature and the enzyme activity was determined. The pumping speed was varied between 1 to 2 ml/min according to the experiment.

2.6.2 Mn^{2+} removal from soluble arginases.

Mn^{2+} removal from soluble arginase was carried out by dissolving the enzyme in 0.02 mole/l Tris-HCl, 0.03 mole/l EDTA, pH 8.0, to a concentration of 5 mg of protein per ml. The enzyme solution was incubated for 40 min at 37° and dialyzed 18 h at 4° against water with 3 changes. After dialysis the enzyme solution was centrifuged at 10,000 g for 10 min to eliminate some precipitated proteins.

2.7 Reactivation kinetics after Mn^{2+} re-addition.

1 volume of EDTA-treated gel was extensively washed with 0.1 mole/l Tris-HCl, pH 7.5 and suspended in the same buffer but containing 0.05 mole/l $MnCl_2$. The suspension was incubated at 37° with constant shaking. At given times suspension samples were withdrawn and activity was assayed.

2.8 Acid-treatment of immobilized arginase.

Arginase-Sepharose was washed two times with two volumes of glycine-HCl buffer, I=0.05, pH 2.7, in a glass filter at room temperature. The gel was then suspended in 2 volumes of the same buffer and incubated at room temperature during 30 min. The suspension was transferred to a glass filter and washed seven times with two volumes of glycine-buffer, pH 2.7, and subsequently extensively washed with 0.1 mole/l Tris-HCl, pH 7.5, containing 0.05 mole/l $MnCl_2$. The immobilized enzyme submitted to such procedure will be referred to acid-treated arginase in the text.

2.9 Assay of soluble subunits binding to immobilized subunits.

2.9.1 Acid-neutral pH shift.

Binding experiments of the acid-dissociated subunits were

performed as follows unless otherwise described. A constant amount of free arginase dissolved in glycine buffer, $I=0.05$, pH 2.7, was mixed with variable amounts of immobilized arginase previously washed with glycine buffer, pH 2.7, as indicated under Sec. 2.8. The suspensions were then neutralized by the following method: The samples at pH 2.7 were transferred to the lower chambers of a dialysis device as illustrated in Fig. 4.

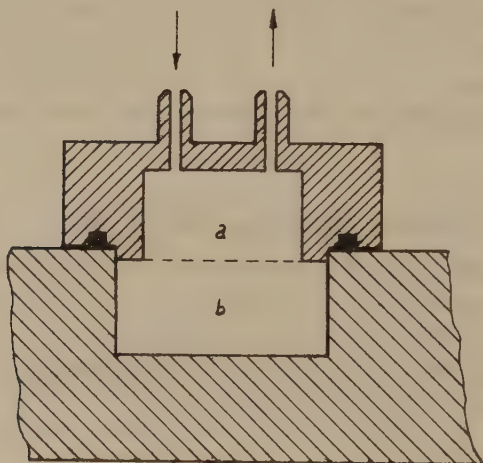


Figure 4. A cross-section of one of the units of the dialysis device. The lower chamber (b) is separated from the upper chamber (a) by a dialysis membrane (dashed line).

The lower chambers were covered with dialysis membranes and the upper chambers were placed on it. The lower chambers were sealed hermetically by pressing them with clamps against the upper chambers. The inlet and outlet of the upper chambers were connected with a buffer reservoir containing 2 l of 0.1 mole/l Tris- HCl, 0.01 mole/l $MnCl_2$ pH 7.5, by a peristaltic pump. The buffer was pumped through the chambers with a flow rate of 4 ml/min and the device was inverted to prevent bubbles remaining in the lower chambers from perturbing exchange through the dialysis membrane.

The dialysis was performed for 2 h at room temperature with gentle shaking of the chambers. After dialysis the contents of the lower chambers were transferred to small graduate test tubes and the pH and total volume were determined. The suspensions were allowed to settle and the supernatant activity was assayed.

2.9.2 Mn^{2+} -induced binding of subunits.

Fractions of EDTA -treated arginase gel were extensively washed with 0.1 mole/l Tris-HCl, pH 7.5, and mixed with variable amounts of soluble arginase previously treated with EDTA as specified under Sec 2.6.2. 1mole/l $MnCl_2$ was added to a concentration of 0.05 mole/l. The mixtures were incubated for 1 h at 37° with shaking and filtered and the gels were repeatedly washed with Tris buffer containing 0.05 mole/l $MnCl_2$. The activity of Sepharose samples was subsequently assayed.

2.10 Kinetic assays.

In experiments with soluble or immobilized arginase, arginine was used at different concentrations, in 0.1 mole/l Tris -HCl, 0.01 mole/l $MnCl_2$, pH7.5, and all the experiments were performed at 25° . The reaction was started by addition of enzyme and followed with a NH_4^+ sensitive electrode in presence of an excess of urease as indicated under Sec. 2.4.1.1. The evaluation of kinetic parameters was done using Eadie-Hofstee plots of initial rate measurements. (EADIE, 1942;HOFSTEE, 1959).

2.11 Temperature dependence of Vmax.

An arginase sample in 0.1 mole/l Tris- HCl, 0.05 mole/l $MnCl_2$, pH 7.5, was incubated at a definite temperature for 5 min and transferred into the reaction mixture equilibrated at the same temperature. The composition of the reaction mixture is specified under Sec. 2.4.1.1 and the arginine concentration was 0.05 mole/l. The temperature was directly controlled in the thermostated reaction vessel. Experiments were carried out in a temperature range of 20° to 35° . The temperature dependence of the electrode response and the inactivation of the coupling enzyme urease were li-

miting factors when trying to extend the studies temperature range to higher temperatures.

2.12 pH-activity studies.

Samples of immobilized enzyme (0.05 to 0.2 ml) suspended in 0.1 mole/l Tris-HCl, 0.01 mole/l MnCl_2 , 7.5 were diluted a hundredfold in 0.1 mole/l Tris-HCl buffer (containing 0.01 mole/l MnCl_2) at different pH values. The suspension was allowed to settle during 1 h and the volume was reduced to 1 ml by removing the supernatant.

In these studies the activity of the immobilized arginases was axceptionally measured as follows: An immobilized arginase sample (1 ml) at a definite pH was preincubated for 3 min at 37° after addition of 0.2 ml of 0.1 mole/l MnCl_2 . The enzyme reaction was started by addition of 0.8 ml of 0.5 mole/l L-arginine at the same pH of the arginase sample. After 10 min incubation at 37° with continuous stirring the reaction was stopped by addition of 0.5 ml of metaphosphoric acid (24% w/v), an appropriate volume was taken for colorimetric urea determination.

2.13 Elution of Mn^{2+} bound to the immobilized enzyme.

The dissociation of Mn^{2+} bound to immobilized rat liver arginase was studied by washing the gel with buffer and testing its activity before and after Mn^{2+} addition.

2 ml of arginase-Sepharose gel were poured into a small column and washed continuously with 0.01 mole/l Tris-HCl, pH 7.5, at 4° . The buffer flow was controlled with a peristaltic pump and maintained with a flow rate of 25 ml/h. At given times the washing was interrupted and gel samples were withdrawn to assay activity with and without Mn^{2+} . For activity measurements with Mn^{2+} the gel sample, suspended in 0.01 mole/l Tris-HCl pH 7.5, was previously activated by incubating it for 10 min at 50° with stirring after addition of 1 mole/l MnCl_2 to a concentration of 0.05 mole/l.

2.14 Glutaraldehyde treatment.

Rat liver arginase immobilized to Sepharose CL 2B was submitted to a cross-linking reaction by glutaraldehyde as follows: A gel volume was extensively washed with 0.2 mole/l phosphate buffer, pH 7.4, and suspended (1:1) in the same buffer. Aliquots of 1 ml of this suspension were incubated overnight with variable amounts of 25% glutaraldehyde at 4° with shaking.

The treated gel samples were washed 3 times with two volumes of phosphate buffer, pH 7.4, and subsequently for several times with 0.1 mole/l Tris-HCl, pH 7.5, and suspended 1:1 in this buffer. From each sample, 0.1 ml was removed to measure activity and the rest was washed with glycine buffer, pH 2.7, and afterwards with 0.1 mole/l Tris-HCl, 0.05 mole/l MnCl_2 , pH 7.5.

2.15 Preparation of rat-beef arginase hybrids.

Beef liver arginase immobilized to Sepharose CL 2B (2 ml packed gel) was washed at room temperature with glycine buffer (I= 0.05, pH 2.7) for several times, suspended 1:1 in the same buffer and a small fraction was taken to measure activity. After addition of 3.5 ml of glycine buffer, pH 2.7, containing 10 mg of rat liver arginase, the suspension was dialyzed overnight in 500 ml of 0.1 mole/l Tris-HCl, 0.05 mole/l MnCl_2 , pH 7.5, at 4°. Then the gel was washed repeatedly with the same buffer and its activity was assayed.

To test the effect of proteolysis on the hybridization capacity of the immobilized enzyme a sample of immobilized beef liver arginase was treated with trypsin according to the following procedure: 2 ml gel were extensively washed with phosphate buffer I= 0.05, pH 7.0, and suspended 1:1 in the same buffer containing 5 mg/ml trypsin. The suspension was incubated for 4 h at room temperature with shaking and afterwards transferred to a small filter and treated as described above in this Section with respect to the acid washing and hybridization procedure.

2.16 The calculation of effectiveness of immobilized arginases.

Most of the results in this work are expressed as enzyme ac-

tivity and its importance is frequently discussed on the assumption that the enzyme activity represents the amount of bound enzyme. The validity of this assumption must be verified since it is a well known fact that in some cases not all of the active enzyme immobilized in a porous support converts substrate with the same efficiency. This phenomenon can be visualized as a diminished substrate availability for the enzyme molecules located in the interior of the porous matrix particle in comparison with the bulk solution.

The magnitude of the substrate gradient inside the matrix, thus the degree of efficiency of the inner enzyme, depends on properties related with:

- The enzyme (kcat, Km, amount)
- The substrate(s) (size, diffusion coefficient)
- The matrix particles (shape regularity, diameter, porosity and pore size)
- The reaction medium (stirring, viscosity).

These parameters consider only the case of a neutral matrix in whose microenvironment the substrate(s) and product(s) present the same solubility as in the bulk solution.

The efficiency of an immobilized enzyme functioning in steady state is characterized by a stationary effectiveness factor (η) defined for a given amount of catalyst:

$$\eta = \frac{\text{rate with immobilized enzyme}}{\text{rate with free enzyme}}$$

The availability of the η value of an immobilized enzyme system makes it possible to evaluate from the measured activity, the enzyme activity as free of substrate diffusion effects.

Generally the diffusional interferences are considered as derived from two kinds of mass transfer phenomena: External (bulk solution to the surface of the support particle) and internal (inside the porous matrix). Mathematical treatments have been presented to estimate the contribution of these two

factors to the substrate gradient inside the support particle. (FINK et al., 1973; MARSCH et al., 1973; KASCHE et al., 1979). The equation for the concentration profile inside of a spherical support particle is of the form:

$$\frac{d^2 c_s}{dz^2} + \frac{2}{z} \cdot \frac{dc_s}{dz} = \frac{R^2 \cdot V_{max}}{\bar{D}_s \cdot K_m} \cdot \frac{c_s(z)}{1 + c_s(z) \cdot \gamma} \quad (2.16.2)$$

in which z represents a point at a distance r from the centre of a sphere with the radius R and is given by the fractional value r/R , $c_s(z)$ represents the substrate concentration at z and is given by the fractional value $\{S\}z/\{S\}_{bulk}$ and $\gamma = \{S\}_{bulk}/K_m$.

Analytical solution of this differential equation is only possible in limited conditions ($c_s \ll K_m$ or $c_s \gg K_m$). A numerical general solution has been presented by KASCHE et al. (1979) using the collocation method developed by VILLADSEN and STEWART (1967). This solution has the advantage that it does not involve boundary conditions that cannot be measured, e.g. the substrate concentration in the centre of the particle. As a result of this calculation, curves are obtained from which it is possible to determine the effectiveness factor as a function of two parameters: a) The Sherwood number (Sh), that represents the contribution of the macroenvironment to the mass transport, and b) the Thiele modulus (ϕ) that represents the contribution of the microenvironment. The physical meaning of these two parameters can be understood by examination of their formulae;

$$(Sh)^2 = 4 + 1.21 \left[\frac{u \cdot 2R}{D_s} \right]^{2/3} \quad (2.16.3)$$

$$\phi = R \left(\frac{V_{max}}{D_s \cdot K_m} \right)^{1/2} \quad (2.16.4)$$

The primed symbols are used for enzyme and substrate proper-

ties in microenvironment and u is the relative velocity of the particle in the solution.

A rather different method for the evaluation of η has been proposed by ENGASSER (1978). The procedure involves the determination of η as a product of two factors: $\eta(\text{external})$ and $\eta(\text{internal})$ which are evaluated separately. Both factors are determined graphically from families of curves for different values of $(S)_{\text{bulk}}/\text{Km}$. A non trivial difficulty arises however, from the consideration that the macro- and microenvironmental effects, from which $\eta(\text{ext})$ and $\eta(\text{int})$ are determined, are not independent from each other (KASCHE et al., 1979) as established by the mass conservation condition (equation 2.16.5)

$$D_s \left(\frac{d(S)}{dr} \right)_{r=R} = \frac{Sh D_s}{2R} ((S)_o - (S)_r = R) \quad (2.16.5)$$

In the present work, η was calculated using both the figures of KASCHE et al., (1979) and those published by ENGASSER (1978) to determine whether or not the reservations expressed above, as to the independent evaluation of $\eta(\text{int})$ and $\eta(\text{ext})$, are justified.

3. RESULTS.

3.1 Rat liver arginase purification.

The purification procedure was carried out with 180 g of albino rat livers. The preparation takes two days including an overnight dialysis and the freeze-drying of the final product. The Table 2 presents the yield and purification of the final product as compared to the crude homogenate.

Table 2: Purification of arginase from rat liver.

Step	Protein amount (g)	Specific Activity (units/mg) ^a	Yield (Activity,%)
Homogenate	43	3.6	100
Ethanol precipitate (after freeze-drying)	0.128	990	83

^aActivity was measured by the method specified in Sec. 2.4.2. but with all solutions at pH 9.5. 1 unit is the amount of enzyme that produces 1 μ mole of urea in 1 min at 37°.

A further characterization of the final product is given in Fig. 5. This shows an electrofocusing experiment in which one part of the electrofocused sample was stained and the rest was used for activity measurements. The location of the activity maximum agrees with the results of HIRSCH - KOLB et al.,(1970) who reported a pI of 9.4 for rat liver arginase.

3.2 Potentiometric activity measurements.

Most of the arginase activity determinations in this work were carried out by potentiometric detection of NH_4^+ produced in the assay medium which contains an excess of urease. The

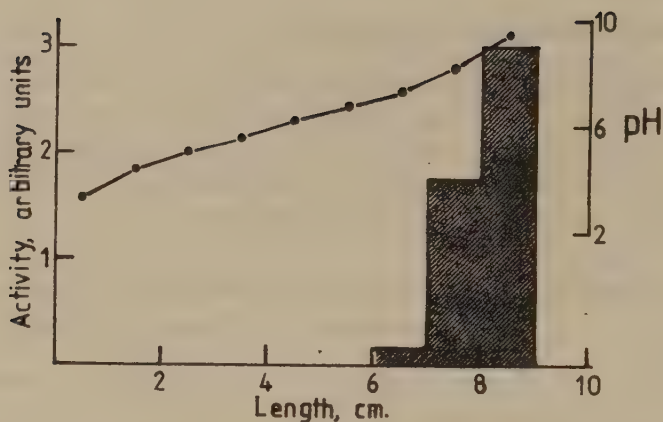


Figure 5. Isoelectric focusing of rat liver arginase. Commercial polyacrylamide gels (PAG plate pH 3.5- 9.5) were focused with a LKB - multiphor at 10° as described in the instruction manual. The photograph shows the protein distribution pattern of a rat liver arginase sample after staining with Coomassie brilliant blue. The activity measurements were done using homogenates of 1 cm wide gel strips cut along the field of 10 samples. Each sample contained about 5 μ l of a 0.2 mg/ml protein solution.

possibility of evaluating arginase activity directly as $\Delta E/\Delta t$ was discussed in Sec. 2.4.1. The following results show the activity ranges in which this assumption has been proven valid. Figure 6 illustrates typical activity measurements for low and high concentration of soluble arginase. Activity measurements were conducted far under the maximal response capacity as shown by the steep slope after urea addition. The slope five minutes after arginase addition was used to make a calibration curve (Fig. 7) for different amounts of soluble, rat liver arginase which activity was previously measured as specified in Sec. 2.4.2 The arrows in Figure 7 indicate the activity range between which the calibration curve was used.

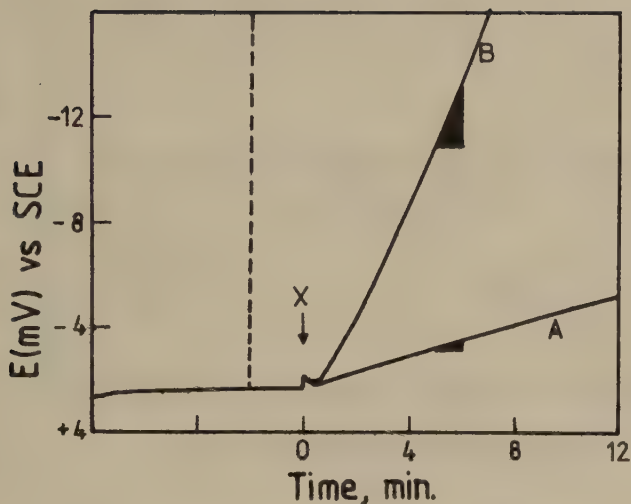


Figure 6. Potentiometric measurements of arginase activity. The response between two concentration limits for rat liver arginase is shown (A: 0.5 units, B: 5.7 units). The arrow (x) indicates the enzyme addition. The dashed line represents the response to 1 mmole/l urea.

3.3 Immobilization of arginase.

Both rat liver- and beef liver arginase were immobilized on Sepharose CL 2B (cross-linked) and 4B, which were BrCN-activated in our laboratory, and 4B supplied by Pharmacia as BrCN-activated Sepharose. The coupling reaction was carried out in all cases at pH 7.0 and 25° for 15 hours.

Table 3 shows the immobilization yield for different matrices determined from the differences between the added amount of protein and the amount of protein washed out of the gel after the coupling procedure.

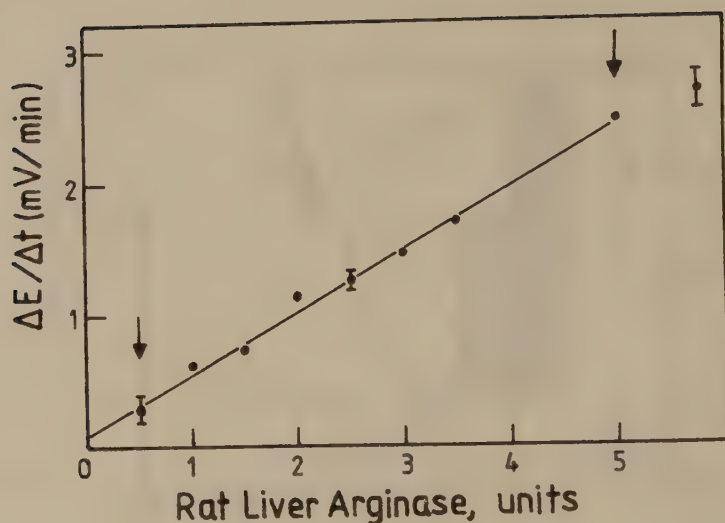


Figure 7. Arginase calibration curve. Each data point was obtained from the slope at the fifth minute (shown as filled triangles in Fig. 6). Potentiometric measurements were made at 25° , pH 7.5 throughout. Bars represent $\pm$ S.D. for n=3

Table 3 Summary of the immobilization of arginases

Enzyme prepara- tion	Sepharose type	Protein yield (%)	Protein bound (mg/ml)	Activity bound (units/ml)
Beef liver	4B	77	0.43	36
arginase	4B ^a	71	0.69	58
	CL 2B	79	0.66	48
Rat liver	4B	40	0.12	180
arginase	4B ^a	69	0.75	700
	CL 2B	50	0.40	300

^a Pharmacia activated Sepharose.

3.4 Effectiveness of immobilized arginases.

The importance of the effectiveness determination was already pointed out in Sec. 2.16 . The values of the relevant matrix and substrate parameters for this estimation are given in Table 4.

Table 4. Parameters for estimation of the effectiveness factor η

Parameter	Value and dimension	Source ^a
Sepharose 4B spheres		
average diameter (dp)	0.011 cm	Microscopic estimation.
Sepharose 4B pore radius (b)	5.0×10^{-9} cm $(4.0 \times 10^{-8}$ cm)	KASCHE et al.(1979) LASCH et al.(1975)
Diffusion coefficient of L-arginine (D)	5.8×10^{-6} cm $\cdot$ s $^{-1}$ (25 $^{\circ}$)	SETLOW (1964)
L-arginine molecular radius	4.0×10^{-10} cm	RENKIN (1954)
Effective D for Arginine in Sepharose (D')	4.0×10^{-6} cm $\cdot$ s $^{-1}$	" "
Sepharose surface per Vol. unit (A)	327 cm $^{-1}$	KOBAYASHI et al. (1973)
Relative velocity of Sepharose particles (U)	0.03 cm $\cdot$ s $^{-1}$	DECHEMA (1979)
Arginine, external transport coefficient.	2.47×10^{-3} cm $\cdot$ s $^{-1}$	ENGASSER (1978)

^aSource from which the value is taken or according to which it is calculated.

In general, for a stirred batch reactor containing immobilized enzyme functioning at high values of $(S)/K_m$, the effectiveness decreases a) with increasing the amount of bound enzyme and b) with increased retardation of substrate(s) and/or product(s) diffusion.

Taking into account these considerations and utilizing the values of the parameters given in Table 4, an example of an effectiveness calculation is presented. For this example an arginase preparation with the highest specific activity (thus the lowest hypothetical effectiveness factor) was taken. Since most of the activity experiments were done at a substrate concentration $[S]$ of 0.05 mole/l this concentration was used for the calculation. At this substrate concentration the activity (v) of the chosen preparation (rat liver arginase coupled to Pharmacia-activated Sepharose 4B) was 0.83×10^{-6} moles urea $\times s^{-1} \times ml \text{ gel}^{-1}$.

For effectiveness calculations according to KASCHE et al. (1979) it is necessary to know the values of the apparent Sherwood number (Sh^*), the square of the Thiele modulus (ϕ^2) and the relative substrate content γ ($= [S]/K_m$). The following results were obtained using these parameters :

$$Sh^* = \frac{D}{2D'} \cdot Sh \quad \text{where} \quad (Sh)^2 = 4 + 1.21 \left[\frac{U \cdot dp}{D} \right]^{2/3}$$

$$\text{from Table 4 ; } Sh = 4.7 \text{ and } Sh^* = \frac{5.8 \times 10^{-6}}{2 \times 4 \times 10^{-6}} \times 4.7 = 3.4$$

For the Thiele modulus calculation:

$$\phi^2 = \left(\frac{dp}{2} \right)^2 \cdot \left(\frac{V_{max}}{D' \cdot K_m} \right)$$

$$\phi^2 = \left(\frac{0.011}{2} \right)^2 \cdot \frac{0.83 \times 10^{-6}}{4 \times 10^{-6} \times 4 \times 10^{-3}} = 1.60 \times 10^{-3}$$

Using a K_m value of 4×10^{-3} mole/l for rat liver arginase, which is based on observations of HOSOYAMA et al. (1972) and my results, $(S)/K_m$ gives a value of 12.5.

For $(S)/K_m = 10$ and the calculated Sh^* and ϕ^2 values, the ap-

plication of the curves presented by KASCHE et al.(1979) produces a η value, which for practical purposes can be considered as 1. Thus, according to this method no corrections are necessary for the present example.

The η estimation method developed by ENGASSER (1978) evaluates separately $\eta(\text{external})$ and $\eta(\text{internal})$. $\eta(\text{ext.})$ is obtained from a diagram of $\eta(\text{ext.})$ versus $v/A \times h \times (S)$, containing different curves for given values of $(S)/\text{Km}$. According to Table 4 and the given enzyme activity, $v/A \times h \times (S)$ has a value of 2.0×10^{-5} and using the curve for $(S)/\text{Km} = 10$, $\eta(\text{ext.})$ is practically 1. For $\eta(\text{int.})$, a graph of $\eta(\text{int.})$ versus an abscissa ratio; $rp^2 \times v/9D' \times (S)_s \times v$ was used, where rp is the radius (average) of the matrix spheres, $(S)_s$ is the substrate concentration at the surface of the matrix spheres. The method of ENGASSER indicates that $(S) \approx (S)_s$ under the present conditions. The abscissa ratio produces a value of 3.48×10^{-6} which location in the curve for $(S)/\text{Km} = 10$ gives a $\eta(\text{int.})$ extremely close to 1. Accordingly, $\eta(\text{ext.}) \times \eta(\text{int.}) \approx 1$.

The method presented by KASCHE et al.(1979) as well as that proposed by ENGASSER (1978) indicate that, in the present case, no corrections are necessary to remove diffusion effects in order to evaluate the actual activity of the immobilized enzyme. This assumption is not true for $(S)/\text{Km}$ values around 1 or lower than 1. From the experiments presented here, the only activity determinations at low values of $(S)/\text{Km}$ were in the kinetic experiments; but also in these cases, effectiveness corrections were not done because low substrate - points are not critical for the estimation of kinetic parameters of immobilized enzymes.

Beef liver arginase also has a low Km (5×10^{-3} mole/l, KUCHEL et al., 1975) and the specific activity of the immobilized preparations is about 10 times lower than that of immobilized rat arginase. Thus, for this enzyme as well no effectiveness corrections were necessary.

3.5 The dissociation of immobilized arginases.

The central questions which led to the immobilization of li-

ver arginase were to determine whether isolated subunits are active and, in that case, is it possible to stabilize them in the matrix in order to further investigate their properties.

Several reports in the last few years account for the suitability of the immobilization approach to study isolated subunits of oligomeric proteins. The general method used to obtain immobilized subunits is: a) coupling of the oligomeric protein to the matrix, b) dissociation of the matrix-bound proteins, c) removal of the released subunits by washing with the dissociating medium. Dissociation is normally carried out in rather drastic conditions, such as 6 mole/l guanidine-HCl (CHAN, 1976), 6 mole/l urea (ANDERSSON and MOSBACH, 1979) or sodium dodecyl sulphate (TAKAHASHI and GROSS, 1978).

Soluble rat- and human liver arginase can be dissociated by removal of Mn^{2+} by chelation with EDTA under relatively gentle conditions (CARVAJAL et al. 1971). Another dissociation method reported for rat liver arginase by HOSOYAMA (1972) is the incubation of the enzyme at pH 2.7. The enzyme treated by both methods can be reactivated and reassociated, in the first case by addition of Mn^{2+} and the second case by restoration of neutral pH. These two methods have been tested using immobilized beef- and rat liver arginase.

3.5.1 Mn^{2+} removal of immobilized arginases by EDTA.

3.5.1.1 Beef liver arginase.

The time course of immobilized enzyme inactivation by EDTA treatment was followed in a closed circulating system. At given times, gel samples were withdrawn and their activity assayed in the presence and absence of Mn^{2+} . Figure 8 illustrates experiments conducted at 22° and 37°.

These results (Fig. 8) show that the inactivation pattern by EDTA is strongly temperature dependent both in the extent of inactivation and in the recoverable activity after Mn^{2+} re-addition. The experiment illustrated in Figure 9 indicates that 1

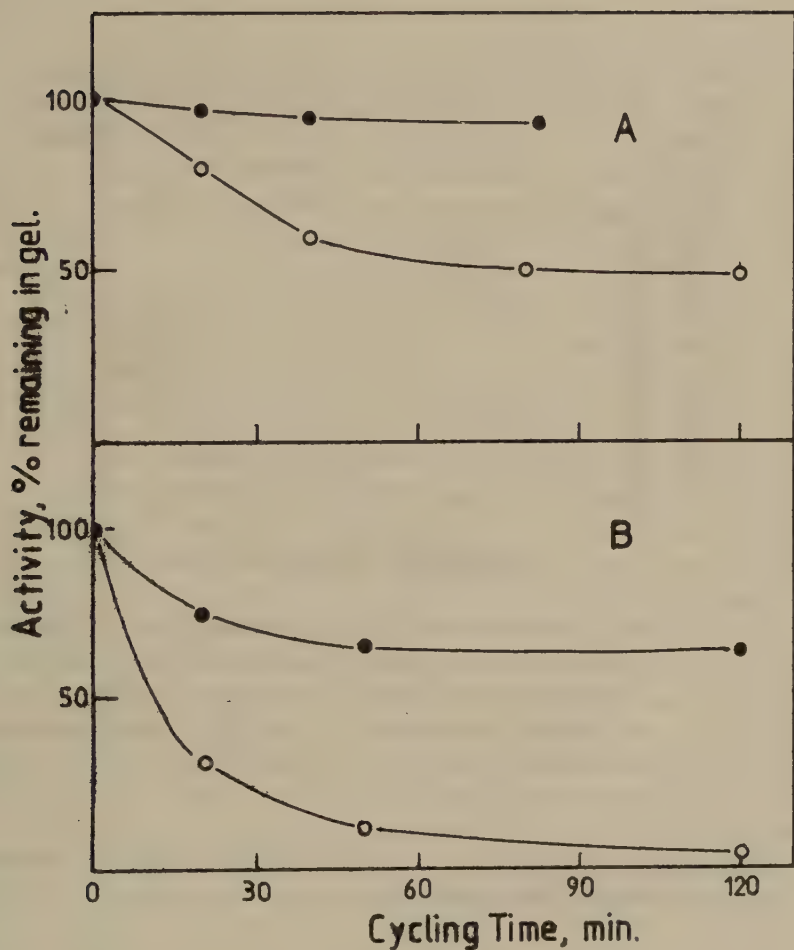


Figure 8. EDTA inactivation of immobilized beef liver arginase. The enzyme immobilized on Sepharose CL 2B was incubated at 22° (A) and at 37° (B) in 0.03 mole/l EDTA, pH 8.0 in a cycling system. The EDTA solution was pumped through the gel at 1.5 ml/min. The inactivation course is represented by the open circles. The filled circles represents the ability of the immobilized preparation to reactivate after Mn²⁺ addition.

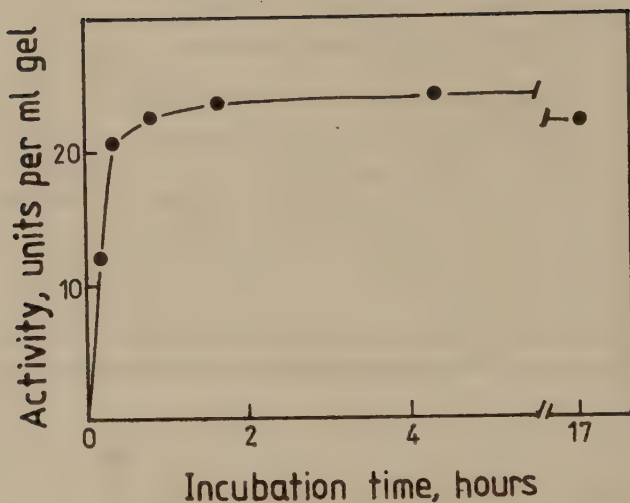


Figure 9. Mn^{2+} -reactivation of EDTA treated, immobilized beef liver arginase. Completely inactivated beef arginase-Sepharose CL 2B was incubated in 0.1 mole/l Tris-HCl, 0.05 mole/l $MnCl_2$, pH 7.5 at 37° and gel samples were withdrawn at the times indicated, in order to assay activity.

hour of incubation with 0.05 mole/l $MnCl_2$ at 37° is enough to reach maximal reactivation.

Assuming that the Sepharose-bound form of the native enzyme is a tetramer and that EDTA causes dissociation into monomers which are capable of reactivation by the addition of Mn^{2+} , the results presented in Fig. 8B could be explained as follows:

a) There are more than one monomer per tetramer bound to the matrix since the recovery after addition of Mn^{2+} is considerably higher than the predicted 25% (see Fig. 2).

b) The specific activity (kcat) of bound, isolated monomers is higher than that of monomers associated in an oligomeric structure.

It is also possible that the original immobilized preparation does not consist of bound tetramers but rather dimers or a mixture of different aggregation states. There are obviously other explanations which are in fact trivial: for example, EDTA treatment does not promote dissociation but rather a discrete irreversible inactivation, or simply an unspecific release of matrix-bound proteins. The latter alternative may be ruled out by virtue of the fact that after EDTA treatment about 87% of the bound protein remains in the gel, as estimated by uv-spectrophotometric determinations, before and after EDTA treatment and washing of the gel. It is necessary to remember that beef arginase is an impure preparation.

In order to gain more information about this system and to test the possibility of specific interactions between free and possibly bound subunits, a reassociation experiment was carried out by mixing constant amounts of immobilized beef liver arginase (Sephacrose CL4B) with variable amounts of free arginase, both previously inactivated by EDTA treatment. The mixtures were incubated in the presence of 0.05 mole/l $MnCl_2$ for 1 h at 37° and pH 7.5. Activities in the supernatant and gel were measured after washing with Mn^{2+} -containing buffer. The results are presented as a Scatchard plot (Fig. 10) where the abscissa parameter (v) has been defined as the number of ligands associated per binding molecule and the ordinate parameter is presented as a ratio indicating the relative saturation of the binding molecule. The extrapolated value of the abscissa intercept represents the maximal number of ligands that can be associated with the binding molecule, thus giving the association stoichiometry of the complex (SCATCHARD, 1949). In the present case, the binding species are represented by the EDTA inactivated, immobilized enzyme and the ligand is the free added enzyme. The parameters of this Scatchard plot were obtained as follows:

$$v = \frac{I_{Ag} - I_{Bg}}{I_{Bg}} \quad (3.5.1)$$

where I_{Ag} is the activity of the gel after incubation with soluble enzyme in the presence of Mn^{2+} and I_{Bg} is the activity of the gel prior to incubation with soluble enzyme (obtained

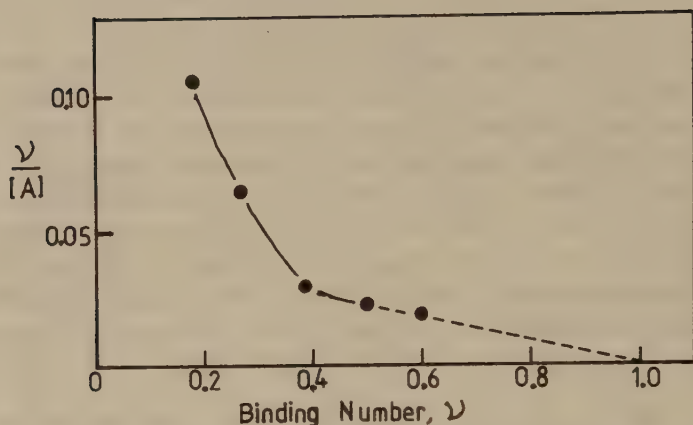


Figure 10. Mn^{2+} -induced association of free, EDTA-treated enzyme subunits to the immobilized form. A Scatchard plot which illustrates the ability of the immobilized enzyme to adsorb free enzyme after Mn^{2+} re-addition. The intercept indicated by the dashed line gives the maximal number of associable soluble molecules per bound molecule.

by incubating a gel sample in Mn^{2+} in the absence of free enzyme). (A) is the activity of the free enzyme in the supernatant.

It is easy to see that if v is 1, the gel has picked up as much enzyme activity of the solution as its own content. To test the extent of non-specific binding, a control was performed using a reference gel without enzyme. No binding was detected in this gel.

Non-linear Scatchard plots, such as that shown in Fig. 10, are normally interpreted as heterogeneous association constants or as a cooperative association process (TANFORD, 1961). In the present case, the interpretation of non-linearity is complicated by the fact that the ligand molecules associate among themselves.

A estimation of the maximal amount of enzyme which could be adsorbed by a given weight of coupled material (thus of the ag-

gregate stoichiometry) can be obtained by extrapolation of the curve to the abscissa. A tangent to a curve drawn through the three last points gives a $v=1$, suggesting a 1:1 complex, although the accuracy of this approximation is indeed rather unsatisfactory (DERANLEAU, 1969).

3.5.1.2 Rat liver arginase.

Immobilized rat liver arginase is considerably more stable than the beef enzyme in regard to the inactivation by EDTA. This immobilized enzyme is also more stable than its soluble form. This stabilizing effect of immobilization has also recently been reported by MUSZYNSKA et al. (1979). A one hour treatment with 0.03 mole/l EDTA at 37° does not produce a noticeable inactivation of Sepharose 4B-immobilized enzyme whereas soluble rat liver arginase loses 50% of its associated Mn^{2+} simply by dialysis against buffer at pH 7.5 (HIRSCH-KOLB et al., 1970).

Even after treatment with 0.1 mole/l EDTA for one hour, the enzyme loses only about 10% of its activity. Figure 11 shows the inactivation kinetics of the immobilized enzyme. Total inactivation is attained after 50 h of 0.1 mole/l EDTA treatment. When Mn^{2+} is added to the completely inactivated enzyme (50 h), the recovered activity is 25% of the original value. These results may favour the assumption that EDTA inactivation produces a dissociation of bound tetramers and that the activity detected in the matrix after Mn^{2+} addition represents isolated subunits.

HIRSCH-KOLB et al. (1970) have reported that the tetrameric form easily loses two Mn atoms while two atoms remain tightly bound. This seems to be a cooperative phenomenon. If as a consequence of EDTA treatment of the immobilized enzyme, isolated bound subunits are obtained, the binding of Mn^{2+} to them will not be subjected to cooperative effects provided that one subunit binds only one Mn atom and that the bound subunits do not interact.

To obtain more information about this process, completely

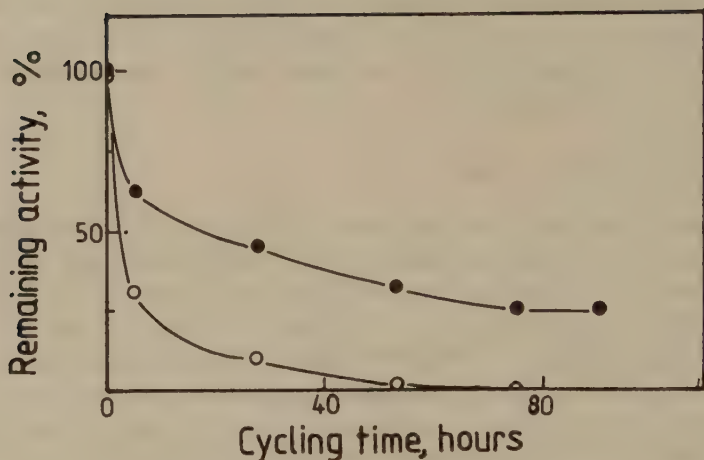


Figure 11. The EDTA-inactivation kinetics of immobilized rat liver arginase. The incubation was performed in 0.1 mole/l EDTA at 37° and cycling flow was 1.5 ml/min. The open circles indicate the activity measured without Mn^{2+} and the filled circles represent that after incubation in 0.05 mole/l Mn^{2+} (1 h, at 37° and pH 7.5).

EDTA-inactivated, immobilized enzyme was incubated with variable concentrations of $MnCl_2$ and the activity recovered in the gel was plotted as a function of Mn^{2+} concentration (Fig. 12A). The sigmoidal pattern observed at low Mn^{2+} concentrations must not necessarily be considered as evidence of cooperative effects or heterogeneity of the Mn^{2+} -binding sites of the enzyme. A plausible explanation for this sigmoidicity is that EDTA remaining in the gel might significantly compete with the enzyme for the metal ion at such low Mn^{2+} concentrations.

It is important to note that with Mn^{2+} at about 5×10^{-5} mole/l, the enzyme seems to be completely active, as deduced from the plateau of the saturation curve (Fig. 12A). A dissociation constant (K_d) for the metal-enzyme can be evaluated upon

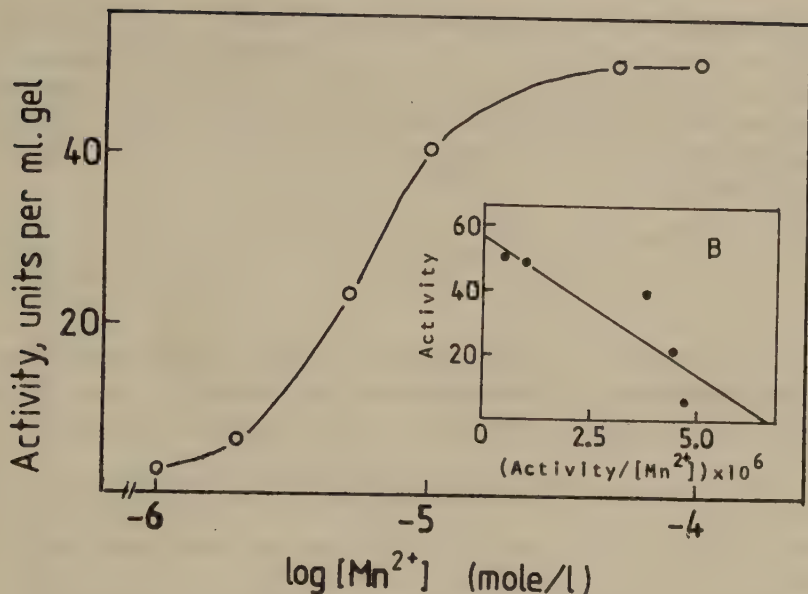
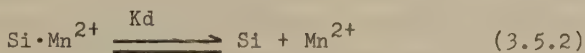


Figure 12. Mn^{2+} -saturation curve of EDTA treated, immobilized rat liver arginase. The gel samples (Sephacrose 4B) were incubated in the indicated Mn^{2+} concentrations at pH 7.5, 37° for 90 min. The activity was determined in the Mn^{2+} concentrations in which the samples were incubated. The slope of Figure B was used to determine K_d .

the assumption that the binding process may be characterized by the simple equation:



where Si is the immobilized subunit, $\text{Si} \cdot \text{Mn}^{2+}$ is the Mn^{2+} -containing, immobilized subunit and;

$$K_d = \frac{(\text{Si}) \cdot (\text{Mn}^{2+})}{(\text{Si} \cdot \text{Mn}^{2+})} \quad (3.5.3)$$

Assuming that Activity = $\kappa(\text{Si} \cdot \text{Mn}^{2+})$ (where κ is a constant), the Mn^{2+} concentration at which half-saturation (half maximal activity) is attained would be K_d since therefore $(\text{Si}) = (\text{Si} \cdot \text{Mn}^{2+})$. By virtue of the considerations expressed above, the hyperbolic section of the curve in Fig. 12 A can be approximated by the expression:

$$\text{Activity} = \text{maximal activity} - K_d(\text{Activity}/(\text{Mn}^{2+})) \quad (3.5.4)$$

The equation 3.5.4 has been represented graphically in Fig. 12 B. According to this equation, K_d is obtainable from the slope of this plot which, thus, has a value of 8.4×10^{-6} mole/l.

The K_d values determined for the soluble tetrameric form by NMR (HIRSCH-KOLB et al., 1971) are 2×10^{-4} mole/l and 3×10^{-7} mole/l for the weakly and tightly bound Mn atoms respectively. Since a plateau is observed already at 5×10^{-5} mole/l (Mn^{2+}) in Fig. 12 A, a weak binding on the order of 2×10^{-4} mole/l, was not apparent in the present work.

EDTA-inactivation of immobilized, rat liver arginase is also possible by dialysis of a gel sample in 0.1 mole/l EDTA at pH 8.0. After 3 days of dialysis at 37° , a completely inactivated preparation was obtained. After Mn^{2+} -reactivation of the washed gel, 25% of the original activity was recovered in the matrix. Attempts to detect enzyme in the supernatant of the dialyzed sample were unsuccessful.

To test the ability of EDTA-inactivated, immobilized enzyme to associate with free EDTA-inactivated enzyme after Mn^{2+} addition, a sample of EDTA-inactivated gel was mixed with an excess (10 times more recoverable activity than in the gel) of soluble EDTA-inactivated enzyme and dialyzed overnight against 0.1 mole/l Tris-HCl, 0.05 mole/l MnCl_2 , pH 7.5 at room temperature. The activities recovered in the gel are summarized in Table 5.

Table 5. Mn^{2+} -reactivation of immobilized enzyme in presence of free enzyme.

Gel without treatment ^a	Gel reactivation in excess of free enzyme	Gel reactivation in absence of free enzyme
100%	60%	25%

^aRat liver arginase-Sepharose 4B=180 Units/ml gel.

3.5.2 The acid dissociation of immobilized arginases.

3.5.2.1 Beef liver arginase.

Immobilized, beef liver arginase is easily inactivated by washing with glycine buffer pH 2.7. After neutralization with 0.1 mole/l Tris-HCl, 0.05 mole/l $MnCl_2$, pH 7.5, activity is recovered in the matrix, but at very low yields. The yield is improved if the gel is neutralized by dialysis in the presence of an excess of free enzyme. These results are given in Table 6.

Table 6. Acid-treatment of immobilized beef liver arginase^a

Immobilized enzyme no treated	neutralized in presence of excess of free enzyme	neutralized in absence of free enzyme
100%	25%	5%

^aSepharose CL 2B preparation.

Owing to the low activity recovered in the gel after neutralization, it is difficult to obtain reliable information about the reassociation stoichiometry based only on enzyme activity. It is possible that immobilized enzyme which is incapable of reactivation, also binds soluble reactivable enzyme to some extent.

3.5.2.2 Rat liver arginase.

When Sepharose-coupled rat liver arginase is washed with glycine buffer, pH 2.7, the activity found in the matrix after neutralization is about a quarter of the originally bound activity. This amount is independent of both the Sepharose type to which the enzyme was coupled and the specific activity of the immobilized preparation. If the acid-inactivated gel is mixed with an excess (10 fold the original gel activity) of free enzyme, dissolved in glycine buffer pH 2.7 and dialyzed in the customary Tris-Mn²⁺ buffer, there is a considerable amount of activity recovered in the gel. These results are summarized in Table 7.

Table 7 Acid-treatment of rat liver arginase.

Conditions	R.L.Arginase Sepharose CL 2B ^a	R.L.Arginase Sepha- rose 4B ^b
Not treated	100%	100%
After acid-washing and neutralization	27%	21%
Neutralization in an excess of free enzyme	85%	78%
^a 320 units/ml gel		^b 700 units/ml gel.

The high yield of reassociated enzyme obtained in the above illustrated experiment suggests that the acidification-neutralization might be a suitable means with which to study the reas-

sociation process in the immobilized enzyme system because reliable results about stoichiometry are obtainable. With this purpose in mind, a reassociation experiment was performed in which pH-inactivated, immobilized- and soluble enzymes were mixed in variable proportions and neutralized by dialysis in small dialysis chambers (for details see Sec.2.9.1). The results are presented in Fig. 13. The parameter v is defined as units of free enzyme associated per unit of coupled enzyme (assumed subunits) after neutralization. The abscissa parameter is the enzyme activity found in the supernatant after neutralization.

Figure 13 resembles a titration curve with at least two affinity constants (TANFORD, 1961). It is interesting to note that an initial plateau is obtained for a v nearly equal to 1 (dimer stoichiometry) whereas at higher free enzyme concentrations, the curve tends to $v = 3$, thus, 3 enzyme units are associated per unit of bound ligand. Assuming that bound ligands are subunits, the association stoichiometry at high concentrations of free enzyme would reveal assembly to tetramers. The two plateaus of the titration suggest an assembly process: monomer $\rightarrow$ dimer $\rightarrow$ tetramer.

According to the thermodynamic equilibrium concept, a small fraction of the subunits remain dissociated at equilibrium even for a tetramer with a high association constant. Accordingly, if soluble tetramers are added to a gel suspension containing coupled subunits, which concentration is considerably higher than the added tetramers, enzyme withdrawal from the solution by binding to the immobilized subunits takes place until a new equilibrium is attained. An experiment confirming this behaviour is shown in Fig.14. A given amount of acid-washed, immobilized enzyme was incubated with diluted soluble enzyme (a tenth of the activity of the acid-washed gel) at pH.7.5 and 4° and the supernatant activity was determined at specified times. The adsorption kinetics seem to be composed of two phases. It is not clear whether the slow process represents non-specific adsorption since the experiment carried out with a reference gel had a similar kinetics. Inactivation was ruled out

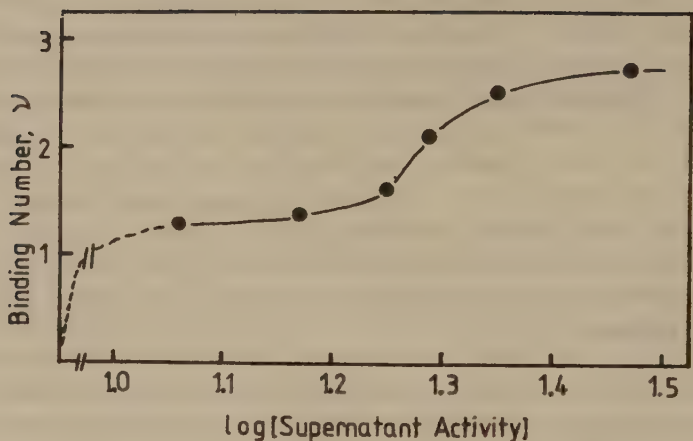


Figure 13 The association of free enzyme with immobilized enzyme after neutralization. A titration-like curve was obtained by mixing acid-washed rat arginase-Sepharose in variable amounts with a constant amount of soluble enzyme at pH 2.7 and subsequently dialyzing the suspensions in neutral buffer. The abscissa parameter represents the soluble activity found after dialysis and is expressed as a logarithm in order to observe the formalism of a titration curve (TANFORD, 1961). The experimental error of v is around $\pm 10\%$.

because the total activity of the sample remained constant.

3.6 Cross-linking by glutaraldehyde.

The assertion that low pH-washing of the coupled enzyme promotes the release of non-covalently bound subunits, led to the prediction that covalent cross-linking between subunits of the immobilized tetramer would prevent activity loss by low pH-washing.

To verify this prediction, native immobilized rat liver arginase was allowed to react with the cross-linking agent glutaraldehyde. This compound reacts with amino groups of proteins by

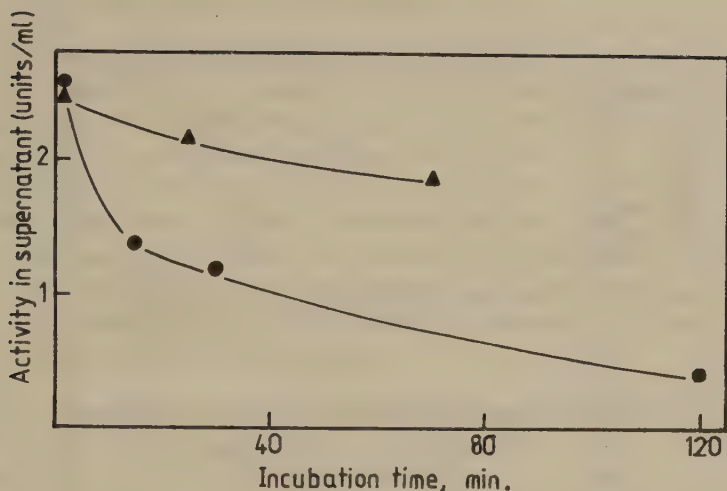


Figure 14 Adsorption kinetics of soluble native rat arginase with acid treated immobilized enzyme. The incubation was done at 4° and pH 7.5 with shaking. The triangles represent unspecific adsorption by a reference gel.

each of its aldehyde groups, forming a bridge between amino groups of intra- and interpolypeptide chains. The reaction is basically a type of Schiff base formation (CORDES et al., 1962).

Figure 15 illustrates the results of cross-linking experiments. Curve A shows that glutaraldehyde treatment considerably inactivates the immobilized enzyme, probably because amino groups important for the enzyme activity are modified. The cross-linked preparations were washed with low pH-buffer and subsequently with neutral buffer and the remaining activity was compared with that found prior to the acid wash. The results are shown in Fig. 15 B. Maximal activity was retained by cross-linking with 2.5% glutaraldehyde. At higher glutaraldehyde concentrations the activity which is retained is somewhat lower, possibly because with an excess of glutaraldehyde each molecule of the cross-linking agent reacts with only one amino group and inter-amino bridges do not occur.

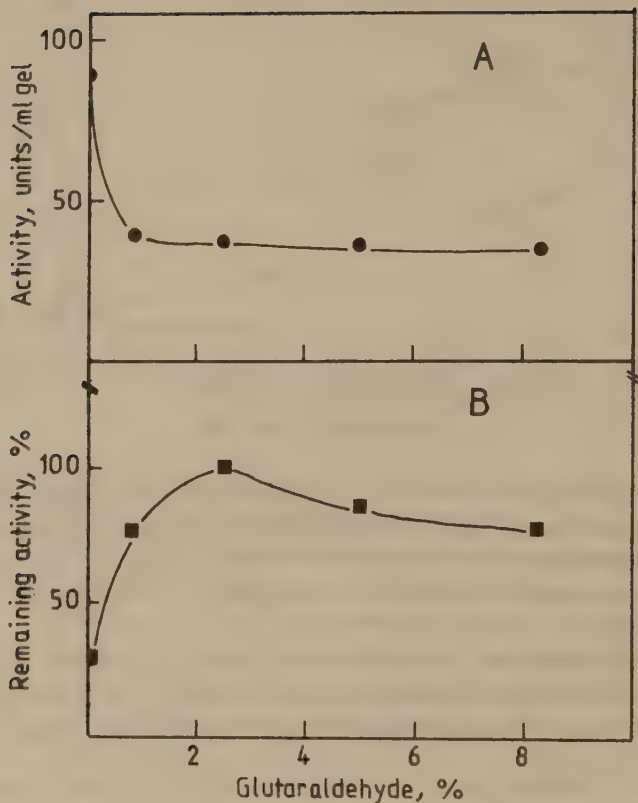


Figure 15 The effect of glutaraldehyde in the acid-dissociation of immobilized rat liver arginase. Figure A shows the effect of the cross-linking agent on enzyme activity. The results of Figure B in terms of percent represent the activity fraction remaining after the acid wash and were calculated as the ratio: activity after acid wash / activity before acid wash (filled circles, 15 A).

3.7 Mn^{2+} dissociation of acid-treated enzyme.

As already stated in Sec. 3.5.1.2, the Mn^{2+} binding of isolated, bound subunits should be free of cooperative effects because subunit interactions are not possible. The free tetrameric form of rat liver arginase has 2 weakly ($K_d = 2 \times 10^{-4}$ mole/l) and two strongly ($K_d = 3 \times 10^{-7}$ mole/l) bound Mn atoms (HIRSCH-KOLB et al., 1971). These two kinds of Mn^{2+} can be easily differentiated by dialysis against buffer at pH 7.5. To compare the affinity of both the immobilized tetramer and the subunit (acid-produced) to Mn^{2+} , matrices containing these forms were washed with neutral buffer.

The tests were done by eluting the gels with Tris buffer, pH 7.5 in small columns, at 4° . Gel samples were withdrawn to assay activity initially and after 1 h incubation in 0.05 mole/l $MnCl_2$ at 37° . The results are presented in Figure 16. Immobilized subunits lose a considerable fraction of its Mn^{2+} very easily whereas the tetrameric form retains most of its Mn^{2+} even after prolonged elution. It is interesting to note that the activity decay in this form is considerably less than the 50% observed by dialysis of soluble tetramers (HIRSCH-KOLB et al., 1970, 1971). This observation is consistent with the results presented in Sec. 3.5.1.2 where a resistance of bound tetramer to EDTA inactivation is developed.

3.8 The characterization of immobilized arginase forms.

The results obtained by EDTA- and acid-treatment of immobilized arginases strongly suggest that after these treatments, active species appear which differ from the original coupled forms. In order to characterize these immobilized species further, some of the treated immobilized enzymes were studied with respect to their K_m , and the influence of pH and temperature on the activity.

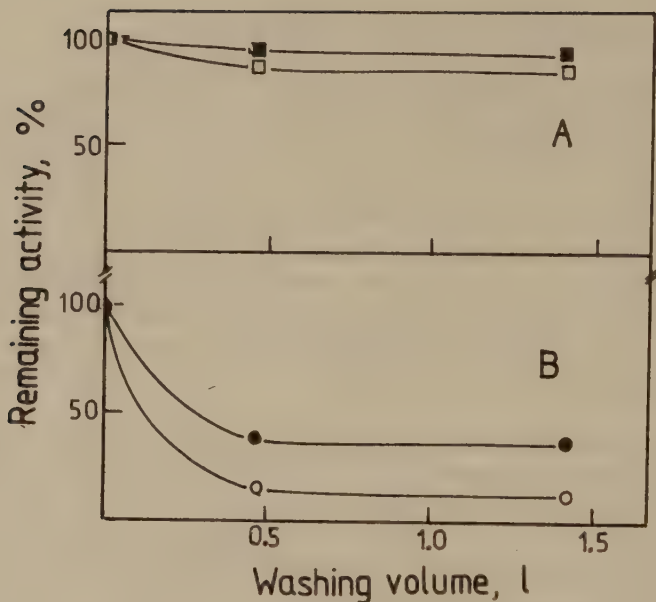


Figure 16 Mn^{2+} -elution of (A) native and (B) acid treated, immobilized rat arginase. Small columns (2 ml gel) were washed at 4° (0.01 mole/l Tris-HCl, pH 7.5) with a buffer flow of 25 ml/h. The open points show the gel activity measured in absence of Mn^{2+} . The filled points were obtained after incubation in Mn^{2+} .

3.8.1 Km determinations.

All the measurements were carried out at 25° and pH 7.5. The reaction rates are given as urea formation ($\text{mole} \times \text{s}^{-1} \times \text{ml gel}^{-1}$). These values were obtained by back calculation from the NH_4^+ production detected by potentiometric measurements.

3.8.1.1 EDTA-treated immobilized arginases.

Experiments were conducted with immobilized enzyme preparations first completely inactivated by EDTA and subsequently reactivated by incubation in 0.05 mole/l $MnCl_2$ at 37° and pH 7.5

for 1 hour. Figure 17 shows the results for beef (A) and rat liver (B,C) arginases. The extrapolated kinetic parameters are given in Table 8.

Table 8. Kinetic parameters of immobilized arginases^a natives and EDTA treated.

Form	K _m (mmole/l)	V _{max} (mole x s ⁻¹ x ml gel ⁻¹)
Beef native	2.2	6.7 x 10 ⁻⁷
Beef EDTA treated	1.6	4.3 x 10 ⁻⁷
Rat native	3.4	8.1 x 10 ⁻⁶
Rat EDTA treated	5.0	1.9 x 10 ⁻⁶

^aBeef liver arginase was immobilized on Sepharose CL 2B and the rat enzyme on Sepharose 4B.

It may be observed that changes in K_m produced by EDTA treatment, if significant, do not occur in the same direction for both enzymes.

K_m values from 1 to 7 mmole/l have been reported for soluble beef liver arginase (KUCHEL, 1975; HIRSCH-KOLB et al., 1970) and from 4 to 20 mmole/l (HOSOYAMA, 1972; HIRSCH-KOLB et al., 1970) for rat liver arginase.

3.8.1.2 Low pH-treated immobilized enzyme.

In this case, the experiments were carried out only with immobilized rat liver arginase. The results are presented in Figure 18. The K_m obtained in this experiment was 18 mmole/l which is significantly higher than the K_m obtained for the untreated form (3.4 mmole/l). It is noteworthy that the EDTA-treated form has a K_m of 5.0 mmole/l. Analogous to the acid treated enzyme this form is also supposed to be an immobilized subunit. The K_m differences between these two forms could suggest different conformational states.

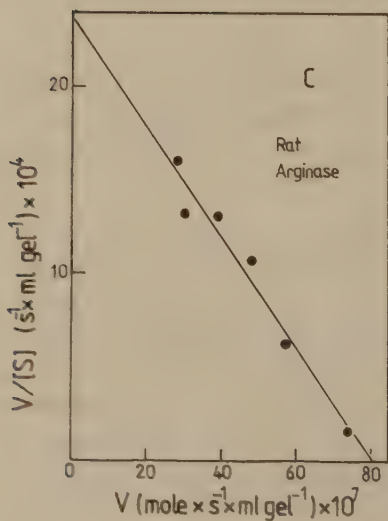
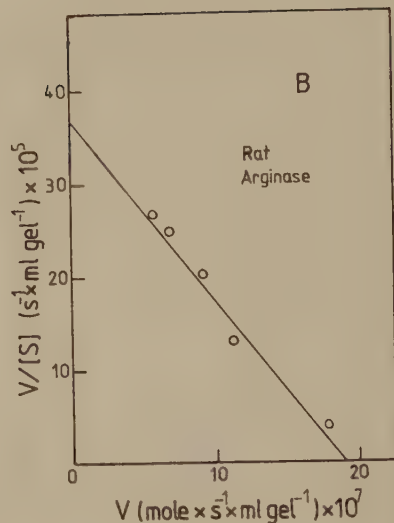
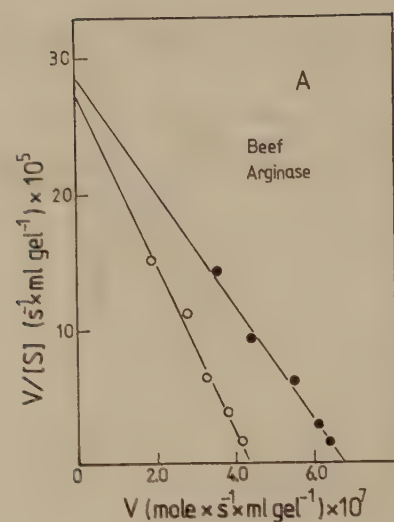


Figure 17.

Kinetic results of immobilized beef (A) and rat (B,C) arginases: the native (●) and EDTA-treated (○). The results are presented as Eadie-Hofstee plots. The abscissa intercept gives V_{max} and the ordinate one gives V_{max}/K_m . The experiments were conducted at pH 7.5 and 25°.

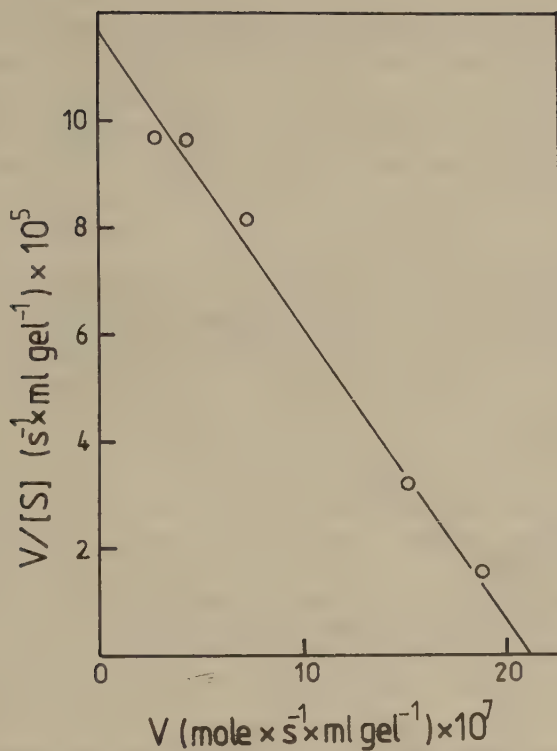


Figure 18. Kinetic results for acid-treated immobilized, rat liver arginase. The conditions are similar to those specified under Fig. 17.

3.8.2 The pH dependence of $V_{\max}$ (kcat).

The effect of pH was studied in rat liver arginase immobilized onto Sepharose 4B and either acid-treated and untreated. The gel samples were incubated for 1 h at room temperature in Tris buffer at different pH before the enzyme reaction was started.

Occasionally, the enzyme reaction was not measured by potentiometry, but rather by the colorimetric determination of urea produced after enzyme inactivation by metaphosphoric acid.

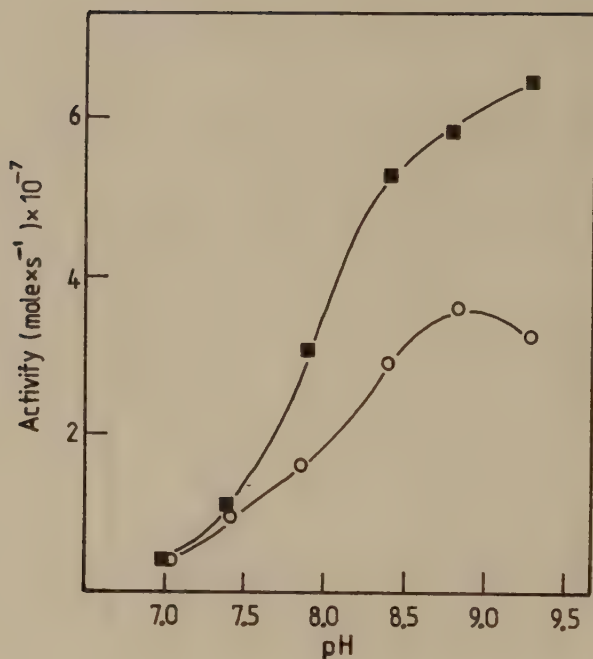


Figure 19. pH-activity profiles for immobilized rat liver arginase. Samples of 0.20 ml of acid treated enzyme (o) and of 0.05 ml of untreated enzyme (■) were used.

The pH optimum detected for the acid treated enzyme (Fig. 19) is about 8.8 whereas that for the native form is over 9.5. The pH optimum for the soluble rat enzyme has been reported as 10 (HIRSCH-KOLB et al., 1970).

3.8.3 Temperature dependence of Vmax.

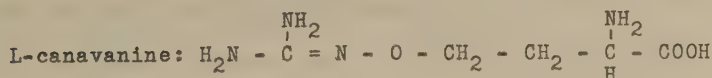
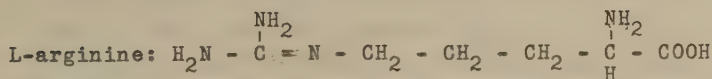
The effect of temperature on the hydrolysis of arginine catalyzed by immobilized arginase was studied as a further differentiating criterion of the tetrameric and monomeric forms of immobilized rat liver arginase. The acid-treated arginase was used as the monomeric form.

Figure 20 shows the results for both immobilized forms as an Arrhenius plot. In the case of the acid-treated preparation, a curve of constant slope is obtained, which gives an activation energy of 5.0 Kcal/mole. The curve corresponding to the untreated species (tetramer), shows two slopes: a steep slope between 20° and 25°, giving an activation energy of 12 Kcal/mole, and a less pronounced one which represents an activation energy of 5.0 Kcal/mole.

The pre-incubation of the acid-treated immobilized enzyme at 37° for 30 min causes a change of the slope between 20° to 25°, as shown by the dashed line. The activation energy obtained from this slope is 8.3 Kcal/mole.

3.8.4 Hydrolysis of L-canavanine.

L-canavanine has also been reported to act as a substrate for arginases (MORA et al., 1965 a,b). Its structure is very similar to arginine:



The hydrolysis of L-canavanine by arginase has been used to characterize ureotelic and uricotelic arginases. The hydrolysis ratio arginine/canavanine of ureotelic arginases is about a fifth of the ratio for the uricotelic enzymes (SOBERON and PALACIOS, 1976).

Table 9 shows the results of L-canavanine hydrolysis by soluble and immobilized rat liver arginases (acid-treated and untreated). The results are presented as hydrolysis ratio (L-arginine/L-canavanine).

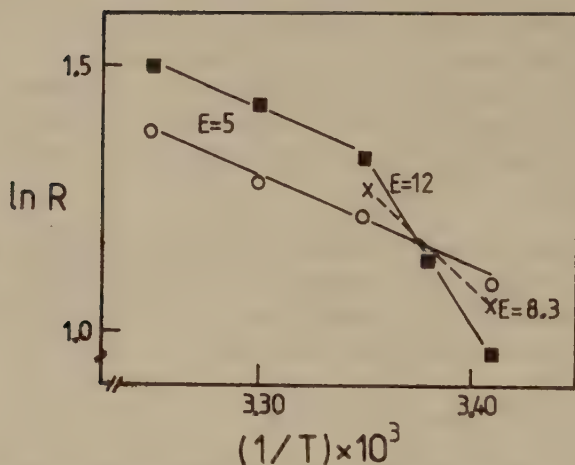


Figure 20 Arrhenius plots for immobilized rat liver arginases. The enzymes were immobilized on Sepharose CL 2B. The immobilized tetramer (■) shows a slope change at 25°. The acid-treated species (○) shows a constant slope but pre-incubation of this form at 37° produces a change in its slope between 20° - 25° (x). The E values represent the activation energy of the corresponding slope. The experimental error of the parameter ln R is around $\pm$ 5%.

Table 9. L-canavanine hydrolysis by rat liver arginase

	immobilized enzyme ^a		
	soluble enzyme	acid-treated	untreated
Hydrolysis ratio (Arg/canav.) ^b	6.5	15.3	13.7

^aArginase-Sepharose 4B (700 units/ml gel).

^bBoth substrates were 0.05 mole/l

The immobilized enzymes show an enhanced specificity for L-arginine. Because of the structural similarities of both substrates, it is difficult to attribute these results to diffusion or partition effects in the matrix. They probably represent intrinsic differences between soluble and immobilized rat liver arginase.

3.9 Subunit exchange chromatography.

The ability of immobilized subunits to pick-up free subunits by displacing the equilibrium towards re-association conditions, offers the possibility of applying this system to enzyme purification. The application scheme can be outlined as follows:

- a) Immobilized subunits are mixed with an impure preparation under dissociating conditions (low pH).
- b) The association conditions are established in the mixture (neutralization).
- c) The subunits are eluted by washing at low pH.
- d) The immobilized subunits may be used again to purify more enzyme by repetition of the steps a, b, c.

Results of an experiment of this type, carried out with rat liver arginase immobilized onto Sepharose CL 2B are shown in Fig. 21. The low pH-elution of the enzyme, which was originally bound, gives an activity peak with a specific activity 3 times higher than the material coupled to the matrix. The low pH-elution of the re-associated material (step c of the purification out-line) produces a peak (Fig. 21B) with a specific activity 4.5 fold higher than the original material. This approximately corresponds to the specific activity of a pure preparation (5000 units/mg, SCHIMKE, 1964).

The ability of the gel to be re-utilized in a new chromatography cycle is illustrated in Fig. 22. The total activity eluted in the first cycle was considered as 100%.

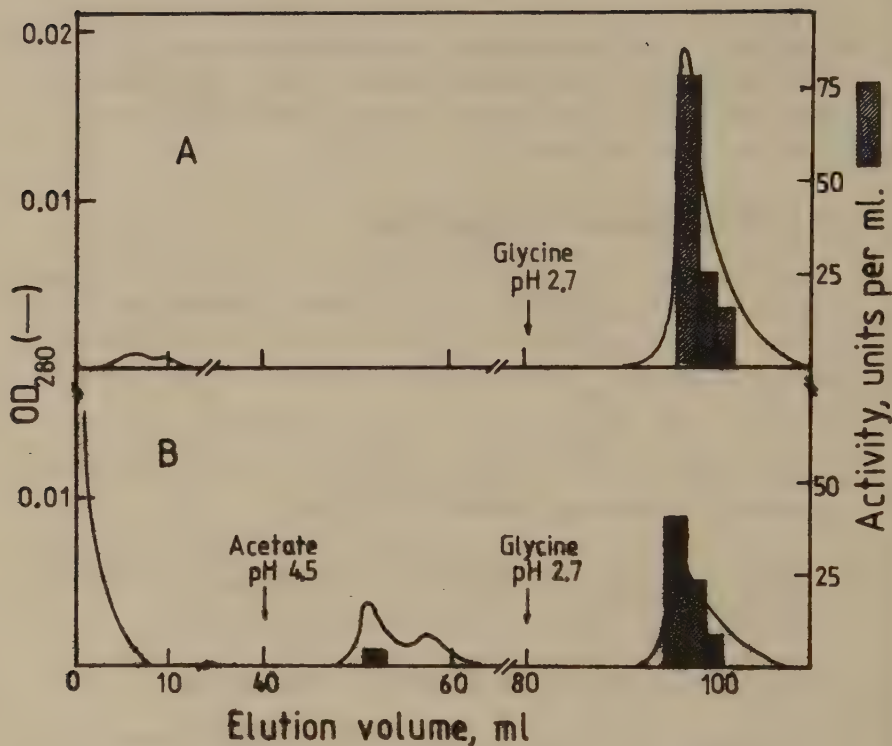


Figure 21. Subunit-exchange chromatography of rat liver arginase.

A) 10 ml of arginase-Sepharose CL 2B was washed with neutral buffer and subsequently with acid buffer (arrow).

B) This material was suspended in acid buffer and 40 mg of free arginase was added. The suspension was dialysed in neutral buffer (with 0.05 mole/l MnCl_2). The gel was poured into a column and eluted successively at 4° with Tris pH 7.5, Acetate buffer ($I=0.05$, pH 4.5) and glycine buffer ($I=0.05$, pH 2.7).

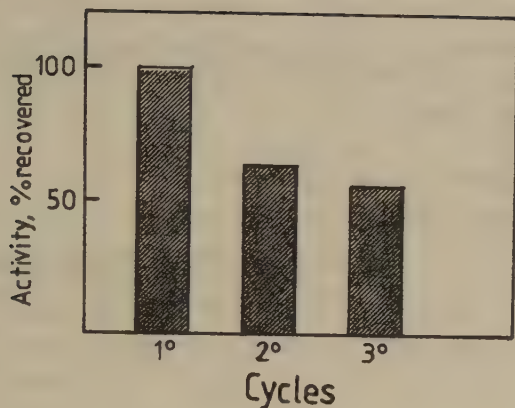


Figure 22. The activity recovered in successive affinity chromatography cycles with the same gel. The total activity eluted at low pH in the first cycle (see Fig. 21B) was considered as 100%.

3.10 The formation of rat-beef hybrid oligomers of immobilized arginase.

The ability of immobilized subunits to bind soluble subunits under associating conditions enables this system to be used to study the formations of immobilized hybrids by mixing immobilized subunits from a given source with free subunits from another one before restoring the association conditions. The resulting immobilized hetero-oligomer can be easily separated from the other components of the reaction mixture.

In the present case, acid-treated beef liver arginase immobilized onto Sepharose CL 2B was used as the immobilized subunits donor and an excess of acid-dissociated rat liver arginase was the free subunits donor. The pH 2.7 mixture was neutralized by dialysis and, after repeated washing at pH 7.5, the activity in the gel was measured. To determine whether the subunits binding capacity of the gel was related to the intactness of immobilized polypeptides, the immobilized enzyme was incu-

bated with trypsin prior to the hybridization experiment. Table 10 summarizes these results, which indicate that trypsin treatment reduces the subunit binding capacity of the gel and that this capacity correlates with the activity of the immobilized subunit donor.

Table 10. Immobilized rat-beef hybrids.

Enzyme form	Activity unit/ml gel	
	Trypsin-treated	not treated
Immobilized beef monomer	0.7	3.2
Immobilized rat-beef oligomer	1.9	9.3

4. DISCUSSION.

4.1. The distribution of bound enzyme in the matrix.

The immobilization procedure, which produces isolated subunits, is based on the assumption that after dissociation of the immobilized oligomeric form of the enzyme, the wide distribution of the coupled subunits makes re-association between them impossible. It is convenient, therefore, to analyse some facts related to the validity of this assumption especially in relationship to the nature of the support material utilized throughout this work (Agarose) and to the density of the attachment sites on the activated matrix.

Agarose is an alternating copolymer of 3-linked β -D-galactopyranose and 4-linked 3,6-anhydro- α -L-galactopyranose residues (ARAKI and ARAI, 1967). Physicochemical studies carried out by ARNOTT et al., (1974) indicated that the copolymer chains are arranged as double helices with a 0.95 nm axial periodicity. Each chain in the double helix forms a left-handed threefold helix of pitch 1.90 nm and is translated axially relative to its partner by exactly half this distance. The authors cited above have proposed that the agarose gel framework is composed of bundles which group 10 to 10^4 double helices. (Fig. 23)

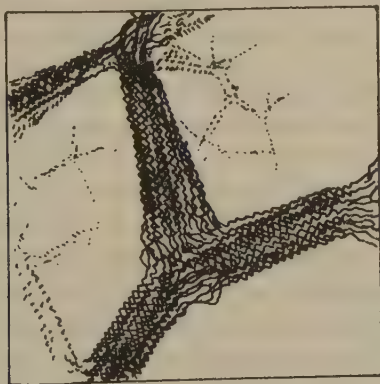


Figure 23. A schematic representation of the agarose network.

Electron microscope observations of Sepharose 4B beads (AMSTERDAM et al., 1975) appear to confirm the framework model proposed by ARNOTT et al., (1974). Randomly distributed pores or channels with variable shape and diameter (up to $0.3 \mu\text{m}$) are observed, and this pattern is not modified by covalent cross-linking of the matrix bundles; indicating a rather rigid structure.

According to this structural model, it is possible that BrCN-activation and the subsequent coupling of proteins takes place preferentially on the surface of the bundles of filaments. Direct electron microscope observations of iron-containing proteins, following BrCN-immobilization to Sepharose reveals that at a particular level of BrCN-activation (30 mg/ml gel) iron spots are regularly distributed throughout the gel matrix (LASCH et al., 1975). At higher activation levels (over 50 mg/ml) a dense spot region is formed at the periphery of the Sepharose beads. This shell-like distribution of bound protein at higher activation levels was interpreted as cross-linkage between polymer chains, partly by interposition of protein molecules occurring in the bead periphery and thereby obstructing access and impeding diffusion. Higher magnification of this densely stained gel shows iron spots concentrated along the matrix bundles and enables measurements of the distance between stained fibres in random directions, which yields an estimate for the mean pore diameter of 84 nm for Sepharose 6B.

The Sepharose matrices used in the present work were of the type 2B and 4B which have a mean pore diameter considerably larger than that of 6B (Pharmacia, information booklet). Assuming a molecular diameter for arginases (M.W. 120,000) of 10 nm, thus probably less than a tenth of the matrix mean pore diameter, it is conceivable that interactions between immobilized arginase molecules might mainly affect enzyme molecules anchored to the same matrix fibre or to contiguous fibres. According to the matrix model presented in Fig. 23, the intersections of bundles represent only a minor fraction of the area available as sites of enzyme attachment.

The degree of BrCN-activation is an important factor which determines both the average density of attached enzymes and the number of attachment sites per enzyme molecule. This factor was first considered in relationship with the immobilization of proteolytic enzymes, where contacts between immobilized enzyme molecules tend to produce autolysis (AXEN and ERNBACK, 1970). With the introduction of immobilization techniques to study isolated enzyme subunits (CHAN, 1970), attention was paid to the density of activated sites in the matrix, since it was desirable to avoid interactions between immobilized subunits. Quantitative relations in this respect have been attempted by GREEN and TOMS (1973). They assumed that the distribution of activated sites is approximately random throughout the Sepharose beads and that the total amount of protein (subunits) to be bound has access to only a small fraction of the matrix volume accessible to BrCN. From these considerations and with a given amount of bound protein, it is possible to calculate the mean volume which contains one protein molecule ($\bar{v}$) and determine the probability (P_n) that a given volume (v) contains n protein molecules. The latter is obtained from a Poisson distribution:

$$P_n = \frac{1}{n!} \left(\frac{v}{\bar{v}} \right)^n e^{-(v/\bar{v})} \quad (4.1.1)$$

P_n can also be interpreted as the fraction of molecules having n neighbours within a volume v . This volume function can be transformed into a distance function:

$$\phi_1 = 1 - e^{-(r/\bar{r})^3} \quad (4.1.2)$$

Where ϕ_1 is the fraction of bound proteins having one or more neighbours within r . $\bar{r}$ is the radius of a sphere which contains on the average one protein molecule and is a function of the molar concentration (c) of bound proteins ($\bar{r} = 7.36 c^{-1/3}$). In a graphical representation of ϕ_1 versus r (or $r/\bar{r}$), families of curves have been obtained for different bound protein concentrations. These curves have a sigmoidal shape and asymptotically approach 1.

tically approach $\phi_1 = 1$ at high values of r . From these curves it may be seen that assuming a bound protein concentration of 1×10^{-4} mole/l all the protein molecules have a neighbour within 300 Å whereas at 1×10^{-7} mole/l only 20% have a neighbour at 1000 Å.

The reported k_{cat} values for rat liver- (SCHIMKE, 1964) and beef liver arginase (HARELL and SOKOLOVSKY, 1972) enable one to make a rough estimation of the enzyme concentration in the gel based on the enzyme activity measurements (Table 3). Under these conditions, the estimated arginase concentrations are around 10^{-6} mole/l in the matrix. According to the corresponding figure of GREEN and TOMS (1973), only about 10% of the bound molecules have one neighbour nearer than 400 Å (approximately four molecular diameters). Therefore, reassociations of two bound subunits derived from different bound oligomers occur rather seldom.

The probability of association between three or four subunits, arising from different bound oligomers, is practically negligible unless the matrix is flexible to a considerable degree. Binding of immobilized subunits occurring between one and the same bound oligomer are possible when the bound oligomer is attached to the matrix by more than one subunit and that would occur when the density of activated sites is too high. Normally this density is considered as a function of the amount of BrCN used in the activation reaction. It is difficult to make assertions about the maximal BrCN concentration which is possible to use without causing multipoint attachment of the oligomer. Differences in the shape, size and binding sites of proteins, before binding, must all be taken into account. Multipoint attachment of the oligomer may be tested by determining if drastic dissociation of a bound oligomer leaves but one of its subunits in the gel. In this respect, Table 11 summarizes some results obtained with different dimeric and tetrameric immobilized proteins. The last column shows the percentage of protein bound to the matrix after dissociation of the oligomer. The numbers in brackets indicate what result might be expected in the absence of multipoint attachment.

Table 11. The effect of activated site density on the multi-point attachment of oligomeric proteins

Enzyme	amount of	Protein content	
	BrCN	immobilized	immobilized
	mg/ml Sepharose	oligomer %	monomer %

Rabbit muscle			
aldolase ^a	1.0	100	24.8 (25)
(tetramer)	5.0	100	30.6
Yeast trans-			
aldolase ^a	1.0	100	64.6 (50)
(dimer)			
Avidin ^b	28	100	50 (25)
(tetramer)	1.6	100	25
D-glyceraldehyde-3P			
Dehydrogenase ^c	4	100	25 (25)
(tetramer)	20	100	25
Alcohol Dehydro-			
genase ^d (dimer)	2.5	100	72 (50)
Glycogen Phosphory-			
lase ^e (dimer)	2	100	50 (50)
Rat liver Arginase ^f			
(tetramer)	20	100	21-27 (25)

^aCHAN (1976); ^bGREEN and TOMS (1973); ^cCHEREDNIKOVA et al. (1980); ^dANDERSSON and MOSBACH (1979); ^eFELD MANN et al. (1972); ^fmy results (in this case the percentual results are referred to enzyme activity).

From Table 11 it may be noted that at low activation ranges (1- 5 mg BrCN per ml Sepharose), the proportion of multipoint attachment is higher for dimers as compared to tetramers.

The BrCN concentration used to immobilize arginases in the present work was 20 mg/ml gel, but probably higher in the case of commercially activated Sepharose. According to the examples of Table 11, these concentrations are high enough to produce a considerable degree of multipoint attachments. However, the specific activity results obtained with rat liver arginase after acid dissociation (Table 7) are quite consistent with the assumption that the tetramers are only bound by one subunit to the matrix, since after dissociation, washing and reactivation a quarter of the original activity remains bound to the matrix. These results are practically independent of the binding capacity of the matrices tested (Sepharoses 4B and CL 2B).

The rat liver arginase preparation which was immobilized to Sepharose was not a highly purified enzyme, but probably contained 80% contaminating proteins. It may be assumed that an arginase tetramer is initially bound at a single site on the surface of a matrix fibres bundle. Subsequent binding interactions may be thought of as falling under two categories: 1) a proximal domain, which includes activated sites that could interact with the already coupled subunit and 2) a distal domain which includes sites capable of reacting with the other subunits. Owing to their closeness, the effective concentration of reactive groups on the bound subunit in the proximal domain is high. Thus, further binding would occur preferentially with the already bound subunit in competition with soluble protein or more remote parts of the bound molecule. A different situation may be assumed for the distal domain in which local excess of other protein strongly competes with the uncoupled subunits of the tetramer for matrix binding sites. This explanation could account for the anomalous behaviour of immobilized rat liver arginase in its multipoint attachment which involves more than one subunit.

An estimation of the extent of multipoint attachment may also be obtained by measurements of protein leakage from the gel (LASCH and KOELSCH, 1978). Immobilized rat liver arginase might conceivably bind to many attachment sites through but one subunit because there is no loss of enzyme activity after the gel is washed with a large amount of neutral buffer (Fig. 16A).

4.2 Arginase: immobilized oligomers and subunits.

In this work, two approaches were used to produce immobilized arginase subunits: 1) washing of the immobilized enzyme with buffer pH 2.7 and 2) chelation of enzyme-bound Mn^{2+} with EDTA. In soluble human- and rat liver arginases, both methods render the subunits inactive (HOSOYAMA, 1972; CARAVAJA et al., 1971), but these are capable of reactivation and reassociation following the restoration of neutral pH or Mn^{2+} addition respectively.

As partly discussed in Sec. 4.1, the acid-dissociation of immobilized rat liver arginase shows that a definite proportion of the original activity is recoverable in the matrix after neutralization. Assuming that dissociation quantitatively occurs in the matrix, the 25% recovery of activity in the gel may indicate that isolated subunits are also active and that they have the same specific activity as subunits which are associated in tetramers. Table 7 demonstrates that free and immobilized subunits re-associate quantitatively. Information about the assembly process after neutralization by dialysis comes from the re-association experiments which were carried out with variable proportions of free/bound subunits (Fig. 13). The shape of the curve shown in Figure 13 suggests a two-step association process: first, association to dimers, in which one subunit is covalently immobilized and the other one originates from the solution; second, subsequent, further association to tetramers. It is not quite clear whether the first plateau indicates a true thermodynamic equilibrium in which the main immobilized species are dimers. The main uncertainty about this equilibrium is that immobilized tetramers do not spontaneously dissociate at pH 7.5 to dimers at a detectable rate,

which is consistent with the equilibrium suggested by the first plateau. That contradicts the reversibility principle required by the definition of a true equilibrium state.

Immobilized active dimers of human liver arginase have been reported by CARVAJAL et al., (1978). These authors dissociated immobilized tetramers to dimers by treatment with p-hydroxy-mercuribenzoate, which does not affect the enzyme activity. Reassociation can be obtained by incubation of soluble and immobilized dimers in the presence of 2-mercaptoethanol. The results of my experiments (presented above), where reassociation is provoked by neutralization of rat liver arginase, as well as those of CARVAJAL et al., (1978) suggest a D_2 symmetry (KLOTZ et al., 1975).

It is predictable that the addition of a suspension of immobilized subunits to a solution of free enzyme under non-dissociating conditions withdraws enzyme from the solution via a free monomer pathway, as represented in Figure 24.

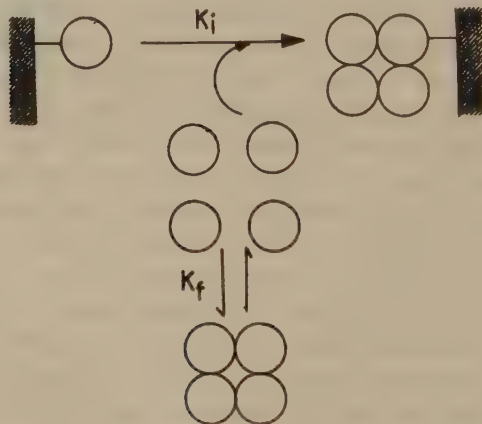


Figure 24. The establishment of a new equilibrium involving formation of immobilized tetramer from free tetramers.

The establishment of this new equilibrium state is demonstrated by the results presented in Figure 14. The fraction of soluble enzyme which is bound by the subunits-containing gel is not only dependent on the concentration of subunits in the matrix but also on the ratio of the association constants K_i/K_f . Recent results with D-glyceraldehyde-3P dehydrogenase (CHEREDNIKOVA et al., 1980) indicate that the soluble dimers of the enzyme preferentially interact with immobilized dimers to form tetramers. This observation may be explained on the assumption that the interdimeric contacts in the matrix-bound tetramer are stronger than those in the soluble form, thus $K_i/K_f > 1$.

The results which I presented above do not allow a direct evaluation of K_i/K_f for rat liver arginase, although the extent of exhaustion of enzyme from the solution effected by immobilized subunits suggests a K_i/K_f larger than 1. This assumption is also supported by the inability of the matrix-bound tetramer to dissociate even after extensive washing with neutral buffer (Figure 16 A).

If the immobilized subunits actually exhibit an enhanced affinity for soluble subunits, it might be possible to selectively extract enzyme from a solution by the mere addition of immobilized subunits and subsequent filtration. This system could therefore offer a very gentle and specific method for arginase isolation and purification.

A separation method, based on the association capacity demonstrated between free and immobilized subunits, was explored by associating free subunits with immobilized subunits by neutralization and thereafter eluting the adsorbed subunits with acid buffer. This subunit-exchange chromatography is shown in Fig. 21. The previous washing of the oligomer-containing matrix with buffer pH 4.5 (Fig. 21B) causes only a small degree of activity release. This finding reveals a significant difference between free and immobilized oligomers of rat liver arginase; whereas the soluble tetramer dissociates quantitatively to dimers at pH 4.5 (HOSOYAMA, 1972), the elution pattern of the immobilized tetramer at this pH does not demonstrate dis-

sociation into dimers. This observation may further substantiate a claim that immobilization of the oligomeric form strengthens inter-subunit bonds.

The acid dissociation of immobilized beef liver arginase (Table 6) shows results which differ quantitatively from those for immobilized rat liver arginase. In contrast to rat liver arginase, the activity recovered on immobilized beef arginase after this treatment (5%) indicates that only a minor fraction of the remaining immobilized enzyme recovers its activity by neutralization. The reactivation of the enzyme is not improved by prolonged incubation in 0.1 mole/l Tris, 0.05 mole/l MnCl_2 , pH 7.5 at 37° .

In view of the results obtained with rat liver arginase, which strongly suggest that immobilized subunits are active after neutralization, the low activity recovered on immobilized beef arginase rather reflects the difficulty of the immobilized beef enzyme subunits to refold in an active conformation. This assumption seems more plausible than the supposition that isolated beef enzyme subunits are not active at all and that the recovered activity represents only the fraction of immobilized subunits which are capable of reassociation between them.

The evidence presented in Table 10 supports the conclusion that the hybridization of arginase is achieved by the mixing of acid-treated, immobilized beef arginase with free rat arginase at pH 2.7 and subsequent neutralisation. Proteolytic enzyme treatment of the immobilized subunit decreases its enzyme activity and its capacity to bind soluble rat enzyme. This indicates that the binding capacity of the gel is related to the conformational state of the immobilized subunits, which determines their enzyme activity. Although the hybridization experiments were done in an excess of soluble enzyme, the immobilized subunits seem not to be saturated with soluble subunits (see footnote¹) which suggests that hybrid formation is not favoured with respect to the reassociation of the homogeneous oligomer of rat liver arginase.

The acid- dissociation of immobilized rat liver arginase may be prevented by cross- linking the immobilized oligomer with glutaraldehyde (Fig. 15). Although in this case, the glutaraldehyde-treatment inactivates the immobilized enzyme (Fig. 15A) to a considerable degree, a cross-linked preparation treated with 2.5% glutaraldehyde retains, after acid-washing, more activity (37%) than a non-cross-linked one (25%).

Some oligomeric proteins spontaneously dissociate upon binding covalently to a matrix (ANTONINI et al., 1975). In the case of immobilized enzymes, this phenomenon leads to partial or total inactivation, depending on whether isolated subunits are active or not. Dissociation may be considerably enhanced

Footnote¹⁾

A rough estimation of the extent of saturation may be advanced, assuming that the enzyme concentration (c) may be represented as enzyme activity (a), as the expression:

$$a = c \times kcat \quad (4.2.1)$$

The activity of an hybrid tetramer (a_t) formed in a medium containing an excess of free rat enzyme and immobilized beef enzyme subunits at a given concentration (c_b) will be:

$$a_t = c_b \times kcat_{(b)} + 3c_b \times kcat_{(r)} \quad (4.2.2)$$

Where $kcat_{(b)}$ and $kcat_{(r)}$ are the catalytic constants of beef and rat arginase respectively. The $kcat_{(r)}$ is 6 fold higher than $kcat_{(b)}$ (SCHIMKE, 1964; HARELL and SOKOLOVSKY, 1972). It may be noted that $c_b \times kcat_{(b)}$ is the activity of the immobilized subunits alone (a_b). Thus:

$$a_t = a_b + 3 \times 6a_b = 19 a_b \quad (4.2.3)$$

In consequence of this the saturation of the immobilized beef enzyme subunits by soluble rat enzyme subunits will be given by the ratio between the observed activity of the immobilized hybrid and a_t . Table 10 gives a value of 15% saturation.

by the customary procedure used to remove proteins non-specifically adsorbed on the matrix. The cross-linking procedure used here to stabilize immobilized rat liver arginase could provide a suitable approach to avoid the dissociation of immobilized enzymes. This procedure might also be valuable as a method of stabilizing hybrids and it has already been used in the immobilization of multi-enzyme complexes (KOCH-SCHMIDT et al., 1975).

The alternative dissociation procedure which I used in the present work, consisted of the treatment of immobilized arginases with the chelating agent EDTA, which dissociates human- (CARVAJAL et al., 1971;1977) and rat liver arginase (BARANCZYK - KUZMA et al., 1976). This treatment gives quantitatively different results for the immobilized forms of rat- and beef liver arginase.

The results of EDTA washes of immobilized beef arginase indicate that the enzyme has at least two kinds of bound Mn^{2+} . A weakly-associated Mn^{2+} is removable by EDTA-washing at 22° (Fig. 8A), whereas strongly bound Mn^{2+} is withdrawn by washing at 37° . According to HARELL and SOKOLOVSKY (1972), the tetrameric form of beef arginase has four Mn^{2+} ions per molecule, three of which are removable by dialysis against water. The dialyzed enzyme containing 1 Mn^{2+} /mole retains 25% of the original enzymatic activity. The catalytic activity seems therefore to be correlated with the number of Mn atoms per molecule. Since EDTA-treatment at 22° reduces the activity of the immobilized beef enzyme to about 50%, it may be assumed that the immobilized tetramer retains two Mn^{2+} per enzyme molecule. However these results are also compatible with the assumption that the immobilized oligomer is a dimer which retains one Mn^{2+} after treatment. This latter possibility is supported by the finding that molecular weight determinations by gel filtration in Sephadex G-200 give a molecular weight of 70,000 for the beef liver arginase preparation used for the immobilization. This molecular weight suggests that a dimer is the most probable aggregation state. If the predominant immobilized species is a dimer and complete inactiva-

tion by EDTA-treatment at 37° (Fig. 8B) produces dissociation of the enzyme into subunits, the activity recovered in the matrix after Mn^{2+} addition (60%) approaches the expected value for immobilized active subunits (50%).

When a mixture of completely EDTA-inactivated soluble- and immobilized beef enzyme is reactivated by the addition of Mn^{2+} , the immobilized enzyme may bind free subunits. The Scatchard plot shown in Figure 10 indicates that the immobilized enzyme binds as much enzyme (activity) as the activity recoverable in the immobilized preparation alone. This result further supports the proposition that the untreated, immobilized species is a dimer which dissociates after complete inactivation by EDTA, producing immobilized subunits which, upon the addition of Mn^{2+} , can be reactivated and which reassociate as dimers when free EDTA-inactivated subunits are present. The failure to detect dissociation of the free enzyme, by gel filtration analysis, after EDTA-treatment, contradicts the above hypothesis and does not allow a definite conclusion to be drawn with reasonable confidence concerning a monomer-dimer association.

The immobilization of pure enzyme ought to be valuable to any further investigation of this topic since, in this case, the determination of protein concentration would parallel enzymic activity and enables one to follow the binding stoichiometry.

The results obtained by EDTA-treatment of immobilized rat liver arginase are quite consistent with the statement that Mn^{2+} -removal promotes dissociation of immobilized tetramers and that the re-addition of Mn^{2+} reactivates the remaining bound subunits (Fig. 11). Thus, EDTA-produced, immobilized subunits are capable of binding free subunits in the presence of Mn^{2+} (Table 5).

By the criteria of K_m (Fig. 17) and pH-optimum (Fig. 19) the immobilized tetramer of rat liver arginase does not show noticeable differences from the soluble form. However, a striking

ing difference has been observed with respect to its affinity for Mn^{2+} . The soluble oligomer easily loses two Mn atoms by dialysis (HIRSCH-KOLB et al., 1971) which are characterized by a K_{diss} of 2×10^{-4} mole/l. The two remaining bound Mn atoms have a K_{diss} of 3×10^{-7} mole/l. The immobilized tetramer loses only a small fraction of its activity after washing with large amount of buffer at pH 7.5 (Fig.16A). Since activity is directly related to the amount of Mn^{2+} bound to the enzyme, the stabilization of the immobilized tetramer indicates that even the weakly-binding Mn^{2+} ($K_{diss} 2 \times 10^{-4}$) is more firmly bound as the result of immobilization. EDTA-treatment antagonizes this stabilization as well; while free enzyme is completely inactivated after only 15 min of incubation in 0.01 mole/l EDTA at 30° , this treatment does not reduce the activity of the immobilized tetramer. MUSZYNSKA et al., (1979) have also recently described stabilization by immobilization using rat liver arginase.

Another manifestation of differences between free and immobilized tetramers is the capacity to hydrolyze L-canavanine. The hydrolysis ratio of L-arginine/L-canavanine is, for both immobilized tetramers and subunits, two fold higher than that of the soluble enzyme. This enhanced specificity for the natural substrate is remarkable if one assumes that the structural changes of the enzyme, produced by immobilization, need not necessarily be related to conformational states of physiological significance. However, one may speculate that the unique properties, detected in the immobilized enzyme, indeed correspond to those of a naturally occurring species, but which are not possible to characterize in solution. Such an hypothesis has been presented by MUSZYNSKA et al., (1979) to explain the enhanced affinity of immobilized rat liver arginase for Mn^{2+} . These authors suggested that free arginase might exist in two or more conformational states and that immobilization might occur preferentially with one of these conformations, shifting the equilibrium toward this species. This hypothetical, naturally occurring form, which might have been stabilized by immobilization has a higher affinity for Mn^{2+}

than the others conformations present in solution. One could complement this argument by adding that the enhanced substrate specificity for L-arginine above L-canavanine is another characteristic of this stabilized (immobilized) form.

The coupling of the enzyme to a Sepharose matrix might therefore mimic the effect of a naturally occurring ligand by displacing the conformational equilibrium toward that of one of the co-existing enzyme forms. This possibility seems to be especially attractive as an explanation of some aspects of the Mn^{2+} affinity of the soluble enzyme. As mentioned above, 50% of the activity of soluble rat liver arginase is dependent on the presence of two Mn atoms characterized by a dissociation constant of 2×10^{-4} mole/l. Therefore, as HIRSCHKOLB et al. (1970) have reported a Mn^{2+} concentration of 5×10^{-2} mole/l is necessary to obtain a fully activated, soluble enzyme. The average concentration of this ion in mammalian liver and plasma has been estimated to be on the order of 1×10^{-5} mole/l (LONG, 1971). This concentration is far below that required to obtain maximal activation of the soluble enzyme.

Some speculations may be advanced to attempt to account for this apparent discrepancy between the requirement and availability of Mn^{2+} : a) There is an intracellular microcompartment containing the enzyme and a high Mn^{2+} concentration; b) The enzyme normally functions at one half of its maximal efficiency; c) The enzyme actually has an higher affinity for Mn^{2+} than that shown in assays with the soluble form. The latter possibility implies that the conformational state with high Mn^{2+} affinity is induced by a ligand. Such a ligand might be absent in the purified enzyme and for this reason the assays made with purified enzyme preparations only involve the enzyme conformation of low Mn^{2+} affinity. The coupling of the enzyme to the Sepharose matrix might counterfeit the effect of a Mn^{2+} -stabilizing ligand.

Mn^{2+} not only activates rat liver arginase, but also protects it against proteolytic enzyme inactivation from dis-

rupted lysosomes (HAIDER and SEGAL,1972). Therefore, it is plausible that the ligand proposed above regulates to some degree the Mn^{2+} content of the enzyme and consequently its vulnerability to proteolytic attack. Since the degradation rate of hepatic arginase can be drastically modified by dietary changes (SCHIMKE et al., 1968), the ligand- Mn^{2+} -mediated enzyme stability suggested above, may be regarded as a regulative mechanism in this respect.

The most pronounced differences between rat immobilized tetramer and its acid-produced subunit were the Mn^{2+} -affinity (Fig.16), K_m and temperature dependence (Fig.18 and 20 respectively). It may be observed that the loss of Mn^{2+} from the immobilized subunit simply, by washing the gel with Mn^{2+} -free buffer, produces a considerable degree of irreversible inactivation. That it is the loss of the Mn^{2+} which causes such irreversible inactivation seems demonstrated by the fact that the presence of Mn^{2+} in the wash buffer prevents any loss of activity.

Different K_m values of the acid-produced and EDTA-produced subunits have been obtained. The differences could indicate that these treatments produce different active conformational states. The question remains unanswered whether these assumed different conformational states are interconvertible. The temperature-dependence experiment (Fig.20) shows that the acid-produced subunits, incubated at 37° for 30 min. have a higher activation energy (8.3 Kcal/mole) than the untreated subunits (5.0 Kcal/mole) in the range of 20° to 25° . The changes in the activation energy produced by incubation at 37° indicate conformational changes in the immobilized subunit. The K_m of the subunits incubated at 37° is indistinguishable from that of the untreated form. Thus, at least under this criteria it is impossible to assert that 37° incubation of the acid-produced subunits causes a transition to the conformation characteristic of the EDTA-produced subunits. The cross-linked matrix used in these experiments (Sepharose CL 2B) is rigid enough to prevent inter-

subunit associations that would eventually occur at higher temperatures (GREEN and TOMS, 1973). The Figure 25 summarizes the results described above, concerning differences between acid- and EDTA-produced subunits.

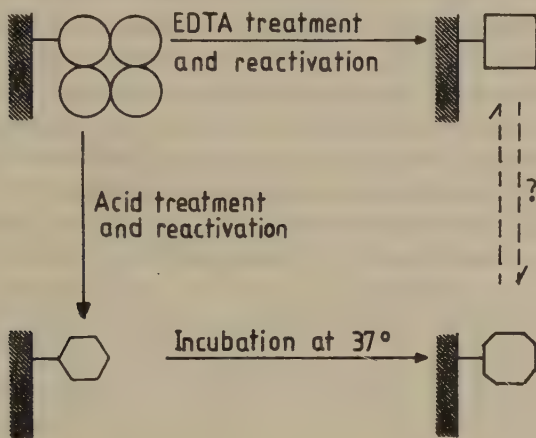


Figure 25. Summary of the probable conformational states of the subunits produced by acid- and EDTA-treatment.

Both the pH- and temperature-dependence experiments (Fig. 19 and 20 respectively) reveal a limitation on the possible determination of the binding stoichiometry of immobilized oligomers based solely on the activity of immobilized subunits. It may be observed from Figure 19 that at pH 7.5 the activity ratio oligomer/subunit for equal amounts of gel is 4.1 which is quite consistent with a tetramer: subunit stoichiometry. However, this ratio is 7.8 at pH 9.3, which represents a considerable deviation from the predicted stoichiometry. In the temperature-dependence experiments (Fig.20) this ratio is 4.2 between 25°-35°, while between 25°-20° it is 2.5.

4.3 The immobilization tool: perspectives.

Questions about the activity of isolated subunits of oligomeric enzymes have received increasing attention in recent years, both as to the elucidation of the physiological role that active subunits might have, and the understanding of the structure-function relationships of oligomeric enzymes. Some approaches, other than immobilization, have been proposed in order to investigate this problem. These alternatives generally include dissociation of the enzyme under denaturing conditions and comparison of the reactivation kinetics with that of the recovery of quaternary structure after establishment of renaturing conditions (JAENICKE, 1978). The application of such methods is sometimes considerably restricted by the experimental conditions which must be chosen (enzyme concentration, denaturing and renaturing conditions) and by the availability of reliable methods to detect protein polymerization and activity without perturbation of the kinetic observations.

The immobilization approach avoids several of difficulties inherent in kinetic methods since the isolated subunits do not undergo association and are separable from oligomers. An additional advantage is that it is possible to produce easily characterizable hybrids by the reassociation of free and immobilized subunits from different sources. In the present work, a beef-rat arginase hybrid has probably been produced (Table 10). The same system might also be applicable to the synthesis of hybrid isoenzyme oligomers (for example, rat-kidney liver arginase). An hybrid, 1 subunit A·3 subunits B, ought to be obtained by saturating immobilized subunits from source A with soluble B-subunits. The inverse order of addition ought to produce a 1B·3A hybrid. An hybrid, 2A·2B, might also be obtained by producing immobilized dimers at first and then subsequently allowing them to be saturated with soluble subunits from the other source. The first plateau of Figure 13 is evidence that immobilized dimers are obtainable by reassociating immobilized subunits with particular concentra-

tion of soluble subunits.

The evaluation of the enzyme activity of immobilized arginases, submitted to different treatments, has produced a considerable amount of information concerning the characterization of isolated subunits and insights into other problems related to the quaternary structure of the enzyme. It seems imperative, however, to complement this information with data about the enzyme concentration. Since estimations of enzyme concentration are not reliable when they are based on enzyme activity and the reported values for k_{cat} (AMNEUS et al., 1976), it is necessary to use highly purified enzyme preparations which thus allow protein concentration to be equal to the actual concentration of the enzyme.

The availability of a pure enzyme would enable quantitative studies of interactions between free and immobilized subunits to be extended to the determination of the equilibrium constants of such interactions. Two methods seem suitable for this purpose. The first has been proposed by ANTONINI et al., (1975) and basically consists of percolating a solution of oligomeric protein through a gel column which contains immobilized subunits. Owing to the interactions between immobilized and soluble subunits, gel-bound subunits will retard the passage of proteins through the gel. The volume difference (ΔV) between protein fronts emerging from columns with and without bound subunits, depends on the protein concentration in the plateau of the emerging front, the column volume and a parameter y . y represents the amount of soluble protein bound to the matrix at equilibrium and depends on the soluble protein concentration (c_s), the concentration of protein bound to the matrix (c_m) and of K_1 and K_2 , which are respectively the association constants of the oligomer in solution and with one immobilized subunit. Given the values of y , c_s and c_m , the values of K_1 and K_2 may be determined. The ratio K_1/K_2 may also be estimated if the equilibria between free-free and immobilized-free subunits are different. This method has been applied to study the association equilibria of hemoglobin and α -chymotrypsin.

The second method of determining the association constant between free and immobilized subunits has been proposed by TAKAHASHI and GROSS (1978) and successfully applied to immobilized chlorophyll a/b protein (tetramer). It basically consists of the quantitative analysis of binding data, as presented in Fig. 13 of this work; but substitutes protein concentration as the abscissa. Computer-fitting of the curve v versus protein concentration allows the determination of the association constants assuming an assembly model: monomer $\rightarrow$ tetramer, or monomer $\rightarrow$ dimer $\rightarrow$ tetramer, as suggested in Fig. 13.

The problem, whether some singular properties of the immobilized rat enzyme, such as its enhanced affinity for Mn^{2+} , are specific properties derived from the particular coupling reaction employed, may be further studied by coupling the enzyme through other reactive groups. A suitable alternative might be coupling through -SH groups. Each subunit of the rat enzyme has two -SH groups and they can be modified without affecting the catalytic activity (SOBERON and PALACIOS, 1976).

The results presented here on the characterization of immobilized arginases may also be considered in relationship to the possibilities of enzyme therapy with arginase in the shock treatment of hyperargininemia cases. Hyperargininemia was first described by TERHEGGEN et al., (1969). This disease, like other congenial disorders of the aminoacid metabolism, not only induces mental retardation but also death in the neonatal period and early infancy (SHIH, 1976). Enzyme replacement therapy has been tried with some relative success, by administration of arginase-loaded erythrocytes to patients with familial hyperargininemia (ADRIAENSSENS et al., 1976). It is conceivable that in acute cases of this disease, an intercorporeal shunt could be temporally implanted with a reactor containing immobilized enzyme. The stabilization of immobilized enzyme against the loss of Mn^{2+} encourages this possibility.

5. SUMMARY

Native rat and beef liver arginases were covalently coupled to Sepharose to characterize the resulting immobilized subunits after dissociation of the matrix-bound oligomer. The dissociation of the immobilized oligomer was performed by washing with pH 2.7 buffer or with EDTA.

When immobilized rat liver arginase is extensively washed with EDTA or acid buffer, the activity recoverable in the gel is a quarter of the value of the untreated immobilized enzyme. This finding is consistent with the assumption that immobilized tetramers have been dissociated and that the remaining activity in the matrix represents the isolated, immobilized subunits. The acid-produced immobilized subunits have a K_m significantly higher than the native enzyme. Mn^{2+} associated to the acid-produced subunits is easily lost by washing with Mn^{2+} -free buffer. The immobilized tetramer does not lose Mn^{2+} by such procedure and its Mn^{2+} -affinity seems to be higher than in the free tetramer. The EDTA-produced immobilized subunits show a K_m slightly higher than the immobilized tetramer, which suggests that acid- and EDTA-produced immobilized subunits are different conformational states.

The immobilized subunits produced by both methods are capable to bind soluble subunits. That allows the application of matrix-bound subunits in order to selectively pick-up soluble subunits from a mixture of proteins, thus to purify enzyme (subunit exchange chromatography).

The results of immobilized beef liver arginase do not allow to assert with reasonable confidence that EDTA-treatment produces immobilized subunits. The activity in the matrix after acid-washing is only 5% of the original value. The enzyme responsible of this residual activity seems to be in the form of immobilized subunits as suggested by its capacity to associate soluble enzyme and to produce rat-beef-hybrids by association of rat subunits.

5. ZUSAMMENFASSUNG

Ratten- und Rindsleberarginasen wurden kovalent zu Sepharose gebunden, um die resultierenden immobilisierten Untereinheiten nach der Dissoziation der in der Matrix gebundenen Oligomere zu charakterisieren. Um die Dissoziation der immobilisierten Oligomere zu erreichen, wurden sie in Puffer pH 2.7 oder in EDTA gewaschen.

Wäscht man immobilisierte Rattenleberarginase wiederholt in Säurepuffer oder EDTA, so beträgt die rückgewinnbare Aktivität im Gel $1/4$ des Wertes der unbehandelten, immobilisierten Enzyme. Dieses Ergebnis stimmt mit der Annahme überein, daß immobilisierte Tetramere dissoziiert wurden und daß die in der Matrix bleibende Aktivität durch die isolierten, immobilisierten Untereinheiten bedingt ist. Die durch Säure hergestellten, immobilisierten Untereinheiten besitzen einen bedeutend höheren K_m als die unbehandelten Enzyme. Das mit den durch Säure produzierten Untereinheiten gebundene Mn^{2+} geht durch das Waschen mit Mn^{2+} -freiem Puffer leicht verloren. Das immobilisierte Tetramer dagegen verliert in einem solchen Vorgang das Mn^{2+} nicht, und seine Mn^{2+} -Affinität scheint sogar höher zu sein als im freien Tetramer. Die in EDTA produzierten, immobilisierten Untereinheiten zeigen nur einen etwas höheren K_m -Wert als das immobilisierte Tetramer, was zur Vermutung Anlaß gibt, daß die in Säuren und EDTA entstandenen immobilisierten Untereinheiten unterschiedliche konformative Zustände einnehmen.

Die durch beide Methoden gewonnenen immobilisierten Untereinheiten besitzen die Fähigkeit, lösliche Untereinheiten zu binden. Das erlaubt die Anwendung Matrix-gebundener Untereinheiten zum Zweck des selektiven Bindens freier Untereinheiten aus einer Proteinmischung: so wird eine Reinigung des Enzyms erreicht (Untereinheiten-austauschschromatographie).

Die Ergebnisse der immobilisierten Rindsleberarginase erlauben nicht, mit nachweislicher Sicherheit zu behaupten, daß eine Behandlung mit EDTA immobilisierte Untereinheiten produziert. Die in der Matrix nach der Säurewaschung wiedergewinnbare Aktivität beträgt nur 5% des ursprünglichen Wertes. Das für diese Restaktivität verantwortliche Enzym scheint in immobilisierten Untereinheiten vorzuliegen; dies wird begründet durch seine Fähigkeit, sowohl lösliche Enzyme zu binden, als auch, in Verbindung mit Ratten- Untereinheiten, Ratten- Rinder Hybride zu bilden.

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